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14. ABSTRACT

Neuroblastoma (NB) is the most common malignant extracranial tumor of childhood. Since NB appears susceptible to immunotherapies that include monoclonal antibodies and T-cell immune responses elicited by tumor vaccine, we have combined the beneficial effects of both humoral and cell-mediated components of the anti tumor response. We demonstrated indeed that adoptive transfer of Epstein-Barr-virus (EBV)-specific cytotoxic T lymphocytes (EBV-CTLs) genetically modified to express a chimeric antigen receptor (CAR-GD2) targeting the GD2 antigen expressed by neuroblasts persist in the peripheral blood and induce objective tumor responses (including complete remissions). We will now augment the expansion and survival of CAR-GD2 modified EBV-CTLs by coexpressing the IL-7Rα that restores their capacity to respond to homeostatic IL-7. We will also enhance the capacity of these cells to invade solid tumor masses by expressing heparanase (HPSE) that disrupts the non-cellular stromal elements of NB. Experiments will be conducted in vitro and in vivo in a xenograft mouse model.

15. SUBJECT TERMS
Neuroblastoma, immunotherapy, chimeric antigen receptor, GD2 antigen, heparanase, regulatory T cells, tumor stroma.

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INTRODUCTION

In our recent Phase I study we found that the adoptive transfer of Epstein-Barr-virus (EBV)-specific cytotoxic T lymphocytes (EBV-CTLs) genetically modified to express a chimeric antigen receptor (CAR-GD2) targeting the GD2 antigen expressed by neuroblasts, can persist in the peripheral blood for 6 weeks and induce objective tumor responses (including complete remission) or tumor necrosis in 4/8 subjects with refractory/relapsed NB[1]. Although encouraging, this study also revealed that the signal from the transgenic CTLs progressively declined over time in the majority of patients[1;2] suggesting that the anti tumor effects of these cells could be augmented by prolonging the survival and effector function of the transgenic CTLs, for example by restoring their responsiveness to homeostatic cytokines such as IL-7[3] and inducing a robust $CD8^+$ T cell memory response[4]. Our second approach aims to disrupt the non-cellular stromal elements of NB that may impede access to CAR-modified EBV-CTLs. The ability of tumor-specific CTLs to cross tumor blood vessels is crucial for reaching the tumor cells. Leukocyte extravasation is highly dependent upon the degradation of the components of the subendothelial basement membrane (SBM) and the extracellular matrix (ECM) such as heparan sulfate proteoglycans (HSPGs), fibronectin and collagen[5]. Heparanase (HPSE) is the only known mammalian endoglycosidase degrading HSPGs at distinct HS intra-chain sites[5;6]. Although HPSE is expressed in activated $CD4^+$ lymphocytes, neutrophils, monocytes and B lymphocytes[5;7;8] we have found it to be deficient in cultured T cells and EBV-CTLs.

BODY

In Task 1 we proposed to co-express CAR-GD2 and IL-7Rα in EBV-CTLs to improve their expansion and anti tumor effects in response to IL-7, whilst avoiding the expansion of regulatory T cells (Treg) (time frame months 1-24).

As indicated in the Progress report 2013 this task has been completed and the results reported in three publications:

1 Chakraborty R, Mahendravada A, Perna SK, Rooney CM, Heslop HE, Vera JF, Savoldo B, Dotti G. Robust and cost effective expansion of human regulatory T cells highly functional in a xenograft model of graft versus host disease. Haematologica. 2013 Apr;98(4):533-7.

2 Perna SK, Pagliara D, Mahendravada A, Liu H, Brenner M, Savoldo B and Dotti G. Interleukin-7 mediates selective expansion of tumor-redirected cytotoxic T lymphocytes without enhancement of regulatory T-cell inhibition. Clin Cancer Res. Clin Cancer Res. 2014 Jan 1;20(1):131-9. Highlited article.

3 Perna SK, Savoldo B, Dotti G. Genetic modification of cytotoxic T lymphocytes to express cytokine receptors. Methods Mol Biol. 2014;1139:189-200.

Task 2. To evaluate the contribution of IL-7Rα ligation and co-stimulation from viral-infected target cells on the development of long-lived memory CAR-GD2-mofied EBV-CTLs in a humanized SCID mouse model previously engrafted with human hematopoietic stem cells (time frame months 12-48).

In the progress report of 2013 we reported the generation and validation *ex vivo* of artificial antigen presenting cells (aAPCs) by engineering K562 to express either CD40L or OX40L or pp65/eGFP or the combinations CD40L/pp65 and OX40L/pp65. We have also reported that aAPCs can boost *ex vivo* CMV-specific CTLs engineered to express the GD2-specific CAR that targets neuroblastoma. As indicated in the previous progress report and illustrated in **Fig. 1**, we continued the task to demonstrate that this approach can be used to boost CAR-redirected CTLs *in vivo* in a xenograft model of neuroblastoma.

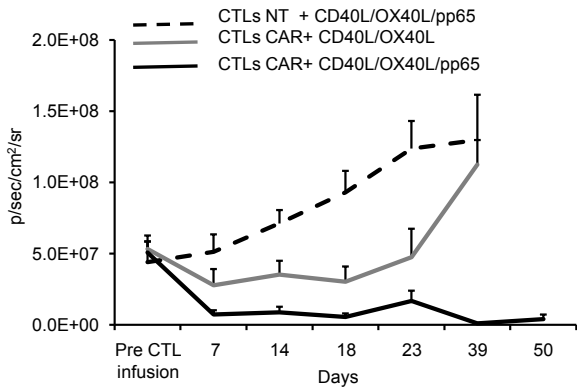

Fig.1, pp65-specific APCs boost CAR-GD2-redirected CMV-specific CTLs in vivo. *NSG mice were engrafted intraperitoneally with the neuroblastoma cell line CHLA-255 labeled with Ffluc and then treated with CMV-specific CTLs expressing the CAR-GD2 and boosted with control aAPC (CD40L and OX40L) or CMV-specific APCs (CD40L/pp65 and OX40L/pp65). The CMV-specific aAPCs induced better antitumor control.*

We are continuing this task by establishing a metastatic tumor model. These experiments will be performed during the one year non cost extension that was recently granted.

Task 3: To co-express CAR-GD2 and HPSE in EBV-CTLs and determine the consequent modulation of NB tissue infiltration and killing (time frame 1-48).

In the progress report of 2013 we reported the accomplishment of the following goals in this task:

- Demonstration that *ex vivo* expanded T cells show reduced invasion of the extracellular matrix (ECM) due to loss of the HPSE.
- Demonstration that p53 down regulates HPSE gene expression by binding to HPSE promoter.
- Demonstration that activated T cells modified to express HPSE reacquire the capacity to degrade ECM.
- Demonstration that HPSE and GD2-specific CAR co-expressed by T lymphocytes retain anti-GD2 specificity and have enhanced capacity to degrade ECM.
- Demonstration that T cells co-expressing HPSE and GD2-CAR have enhanced antitumor activity in the presence of ECM in two models of neuroblastoma (LAN-1 and CHAL-255 tumor cells) LAN-1 cell line.
- Demonstration of enhanced tumor infiltration by CAR-GD2^{+}HPSE^{+} LTE-T cells in orthotopic xenogenic mouse model.

As stated in the previous progress report these data were summarized in a manuscript submitted to Nature Medicine. We received the comments from the reviewers in May 2014 and we are currently addressing the reviewer's comments. In particular we are conducting the following experiments:

We are evaluating whether the constitutive expression of HPSE is toxic in mice. We conducted experiments to determine that the tissue biodistribution of T cells is not altered by HPSE expression (**Fig.2**). We are also conducting experiments in which mice are euthanized at the first passage after the infusion of T cells to evaluate if any immediate toxicity in lung or liver occurs.

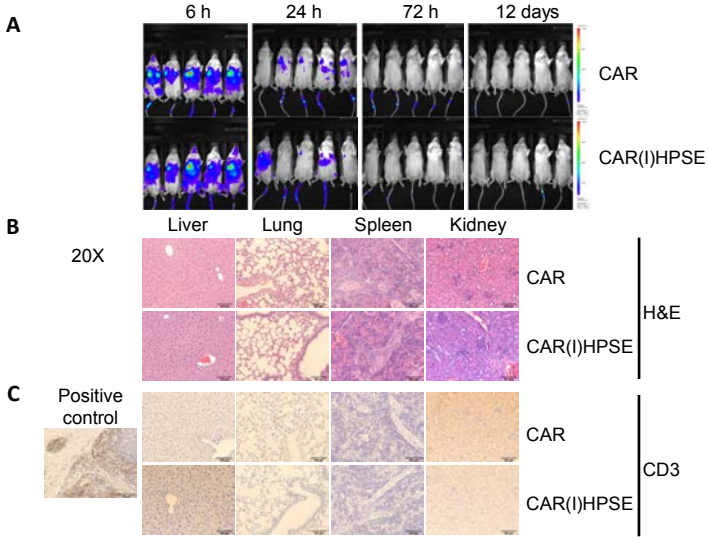

Fig.2 Re-expression of HPSE does not affect T-cell biodistribution in vivo. *CAR(I)HPSE^{+} and CAR^{+} T were labelled with the vector encoding GFP.FFluc and then infused via tail injection in NSG mice. T-cell biodistribution was evaluated by in vivo imaging at indicated time points after T-cell infusion (**Panels A**). Tissues were collected from infused mice by day 12 or 19 after T-cell infusion and stained with haematoxylin and eosin (**Panels B**) and anti-CD3 antibody (**Panels C**). 20X magnification. Human tonsil sections were used as positive control for CD3 staining.*

One reviewer was wondering if the strategy we propose may also have an impact in hematological malignancies. We thus are conduction experiments *in vivo* in a xenogenic transplant model of leukemia and T cells engineered to express a CD19-specific CAR. As illustrated in **Fig.3**, the first set of experiments shows that, since CAR19-T cells are already effective in eliminating the leukemia, the addition of HPSE is not necessary. This further underlines our original hypothesis that our approach is highly relevant for solid tumors such as neuroblastoma. We will continue this task by confirming the results illustrated in **Fig.3**.

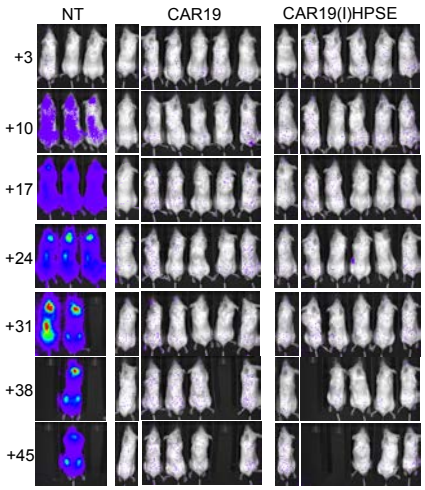

Fig.3. HPSE over expression in T cells does not play a role in a leukemia model. *NT, CAR19 and CAR19(I)HPSE^{+} T were infused via tail injection in NSG mice engrafted with Daudi CD19^{+} tumor cells labelled with GFP.FFluc. Tumor growth was evaluated by in vivo imaging at indicated time points after T-cell infusion.*

Reviewers also requested to assess whether HPSE re-expression is stable in transduced T cells. We addressed this point by collecting T cells infused into the mice 30 days after the infusion and then analyzed the expression of HPSE in these cells by Q-PCR. As illustrated in **Fig.4**, we found a consistent expression of HPSE only in mice infused with T cells co-expressing CAR and HPSE.

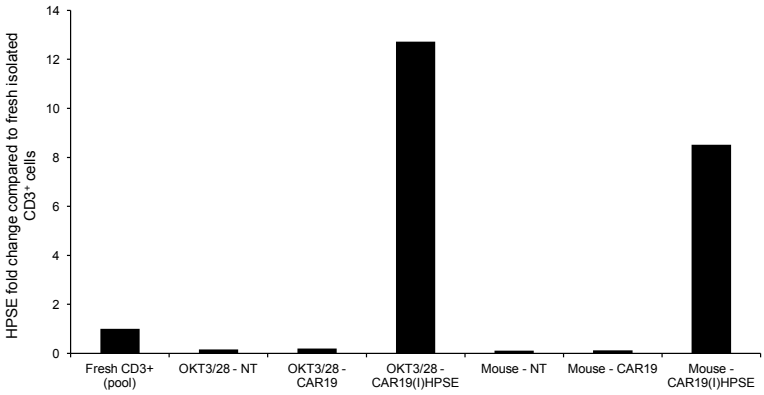

Fig.4. Transgenic HPSE remains over expressed in T cells circulating in vivo in mice for 30 days. *NT, CAR19 and CAR19(I)HPSE$^+$ T were infused via tail injection in NSG mice engrafted with Daudi CD19$^+$ tumor cells labelled with GFP.FFluc. Thirty days after T cells infusion mice were euthanized to collect the spleen and select human CD45$^+$ cells. Q-PCR was performed on the mRNA isolated from these cells to asses the expression of HPSE.*

Reviewers also asked whether constitutive expression of HPSE is equally down regulated in T cells cultured using different experimental conditions and whether these cells are functionally affected. We are performing experiments showing that T cells expanded in human AB serum rather than FBS or using IL-7/IL-15 cytokines rather than IL-2 or high doses of IL-2 rather than low doses of IL-2 do not modify the outcome of HPSE downregulation that we have observed. Other experiments that must be conducted to address other reviewers comments are: (1) analysis of metalloproteases to assess whether these enzymes show similar down regulation in T cells as observed for the HPSE and (2) experiments in vitro and in vivo to show whether T cells expressing a different CAR targeting another antigen expressed by solid tumor show similar outcome as observed for the GD2 antigen expressed by neuroblastoma. We will address this point by making use of the CAR that target the chondroitin sulphate proteoglycan 4 (CSPG4) that we have recently published[9]. All these experiments will be performed during the one year non cost extension that was recently granted.

KEY RESEARCH ACCOMPLISHMENTS

- We optimized the methodology to expand *ex vivo* fully functional regulatory T cells (Tregs) (as assessed in a model of graft versus host disease (GvHD)) that can be used for the experiments *in vitro* and *in vivo* proposed in this task of the proposal. The results of these experiments have been published in manuscript #1 listed in the Reportable Outcome section (Chakraborty R et al. Haematologica. 2013 Apr;98(4):533-7).
- We formally demonstrated that our proposed hypothesis that the genetic manipulation of EBV-CTLs to express CAR-GD2 and IL-7Rα renders these cells resistant to the inhibitory effects of Tregs *in vitro* and *in vivo* in a xenograft neuroblastoma model is correct. The results of these experiments have been summarized in manuscript #2 and #3 listed in the Reportable Outcome section (Perna SK et al Clin Cancer Res. Clin Cancer Res. 2014 Jan 1;20(1):131-9. Highlited article.
- We have generated artificial antigen presenting cells (aAPCs) that express pp65, CD40L and OX40L efficiently boost *in vitro* virus specific CTLs.
- We have demonstrated that these engineered aAPCs boost *in vivo* virus specific CTLs in a xenograft model.
- We have demonstrated that engineered aAPCs boost *in vitro* virus specific CTL redirected with a CAR that targets the GD2 antigen expressed by neuroblasts.
- We have demonstrated that HPSE is defective in *ex vivo* expanded T cells, but it can be restored by retroviral gene transfer and this improves T-cell invasive capacity.
- We found that HPSE down regulation in activated T cells is mediated by p53.
- We have generated a bicistronic vectors that functionally encodes both HPSE and CAR-GD2 that targets neuroblastoma. We found that T cells genetically modified with this novel vector show enhanced invasion of the cellular matrix.
- We demonstrated that T cells coexpressing HPSE and CAR-GD2 promote better anti-tumor activity in two xenograft models of neuroblastoma. These results have been summarized in manuscript #3 (Caruana et al, manuscript in revison for publication in Nature Medicine).

REPORTABLE OUTCOMES

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CONCLUSIONS

From Task 1 we have demonstrated that our proposed approach to modulate the IL-7/IL-7Rα receptor axis in EBV-CTLs redirected with a CAR that targets the GD2 antigen expressed by neuroblastoma promotes the expansion of these cells in response to IL-7 without favoring the expansion of Tregs. This is highly relevant since this strategy will support better expansion of these cells in patients with neuroblastoma without promoting Tregs that are particularly abundant in these patients and significantly contribute in blocking immune responses.

From Task 2 we have generated artificial antigen presenting cells aAPCs that can boost virus-specific CTLs expressing a CAR-GD2 specific. We will continue to validate these aAPCs in a xenograft model of neuroblastoma. If the experiments are successful, this represents another relevant strategy that can be added to the one described in Task 1 to promote the survival on CAR-redirected CTLs in patients with neuroblastoma.

From task 3 we have discovered a major deficiency of T cells used for adoptive immunotherapy. These cells lack the expression of a key enzyme – HPSE – that drives their infiltration of the stroma of solid tumors. We also demonstrate that this defect can be repaired enhancing the capacity of these cells to eliminate neuroblastoma cells in a relevant xenogenic mouse model. This approach may also play a crucial role in improving the clinical efficacy of CAR-redirected CTLs.

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Immunological Reviews

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Design and development of therapies using chimeric antigen receptor-expressing T cells

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Summary: Investigators developed chimeric antigen receptors (CARs) for expression on T cells more than 25 years ago. When the CAR is derived from an antibody, the resultant cell should combine the desirable targeting features of an antibody (e.g. lack of requirement for major histocompatibility complex recognition, ability to recognize non-protein antigens) with the persistence, trafficking, and effector functions of a T cell. This article describes how the past two decades have seen a crescendo of research which has now begun to translate these potential benefits into effective treatments for patients with cancer. We describe the basic design of CARs, describe how antigenic targets are selected, and the initial clinical experience with CAR-T cells. Our review then describes our own and other investigators' work aimed at improving the function of CARs and reviews the clinical studies in hematological and solid malignancies that are beginning to exploit these approaches. Finally, we show the value of adding additional engineering features to CAR-T cells, irrespective of their target, to render them better suited to function in the tumor environment, and discuss how the safety of these heavily modified cells may be maintained.

Keywords: *cancer, immunotherapy, T cells, CAR, gene therapy*

Introduction

Chimeric antigen receptor-expressing T (CAR-T) cells are examples of adoptive cellular immunotherapies (ACIs) which are themselves a subset of complex biological therapies (1, 2). While such therapies have been available for more than 20 years, it has proved difficult to develop them to a stage at which they can be predictably successful and widely implemented as a standard of care. In this review, we outline some specific approaches to overcome these barriers for CAR-T cells, but it is important also to understand the more general barriers that ACIs face in becoming approved therapies in clinical practice.

Many ACIs have to be individually made for each patient, a challenge to the robust scalability required for late phase clinical studies. Moreover, the standard pharmaceutical business model is to recoup the costs of initial drug development

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by selling cheap-to-manufacture licensed drugs that ameliorate rather than cure and that are administered over a prolonged period of time with exceedingly high profit margins. Many ACIs will remain expensive to produce even after approval, an effect compounded by the stacked license fees for the many patents covering the multiplicity of intellectual property incorporated in a single product. Unlike many conventional drugs, ACIs, including CAR-T cells, are intended to be curative not ameliorative, so that they will need to be given once, or a few times, only. Finally, the very specificity of these ACIs means that only a small subset of patients with any given cancer may be suited to treatment, making every ACI an orphan drug. In combination, these market issues can lead to an unaffordable pricing structure with little appeal to pharmaceutical companies.

In this review, we describe how we are developing a 'plug and play' approach to adoptive immunotherapy, using CAR-T cells directed to cancer. With this approach, it will be possible to use a multiplicity of genetic engineering strategies that can enhance access to and killing of many different types of tumor cells. The tumor targeted is then altered simply by changing the specificity of the targeting receptor on the adoptively transferred effector T cells. In other words, broadly applicable strategies will be made specific for individual tumors by coupling the engineered T-cell to specific CARs.

For this concept to work, we must first define appropriate target antigens within and around tumor cells to provide specificity of action, and devise receptors that can signal to the T cell that it has engaged the appropriate antigen within the tumor or its microenvironment. We have to then develop generic approaches to enhance the anti-tumor effector activity of the adoptively transferred cells, increase their resistance to tumor immune evasion strategies, and allow the immune response to be terminated should it prove toxic or damaging to the host immediately or in the longer term.

Design of the chimeric antigen receptor

CARs combine the antigen-binding property of monoclonal antibodies with the lytic capacity and self-renewal of T cells and have several advantages over conventional T cells (3–5). CAR-T cells recognize and kill tumor cells independently of the major histocompatibility complex (MHC), so that target cell recognition is unaffected by some of the major mechanisms by which tumors avoid MHC-restricted T-cell recognition, such as downregulation of human leukocyte antigen class I molecules and defective antigen processing.

Chimeric immune receptors were first developed in the mid-1980s and initially consisted of the variable (antigen binding) regions of a monoclonal antibody and the constant regions of the T-cell receptor (TCR) α and β chains (6). In 1993, Eshhar *et al.* modified this design to use an ectodomain, from a single chain variable fragment (scFv), from the antigen binding regions of both heavy and light chains of a monoclonal antibody (*Figs 1 and 2*), a transmembrane domain, and an endodomain with a signaling domain derived from CD3-ζ. Most CARs subsequently designed and used have followed this same structural pattern, with incorporation of co-stimulatory signaling endodomains, which are described in *Fig. 1* and below (Provision of Co-stimulation to enhance T-cell activity after antigen-specific receptor engagement). In this section, we describe how the composition of the *ectodomain, hinge,* and *transmembrane domain* influences CAR function and the consequent behavior of the T cell that expresses it (*Fig. 2*).

Ectodomain of CARs

ScFvs are the most commonly used ectodomains for CARs, and the affinity of the scFv predicts CAR function (7, 8). For example, T cells expressing CARs containing high affinity ROR1-specific scFv have superior effector function than low affinity scFvs (7). There is, however, a plateau above which further affinity maturation does not increase T-cell activation for any given CAR. The likely explanation for the plateau effect is that the avidity of the CAR needed for maximal T-cell activation is a function of the number and density of the expressed receptors as well as their affinity (8). In addition to CAR affinity, function is also affected by the location of the recognized epitope on the antigen (9, 10). For example, CAR-T cells expressing an scFv that recognized

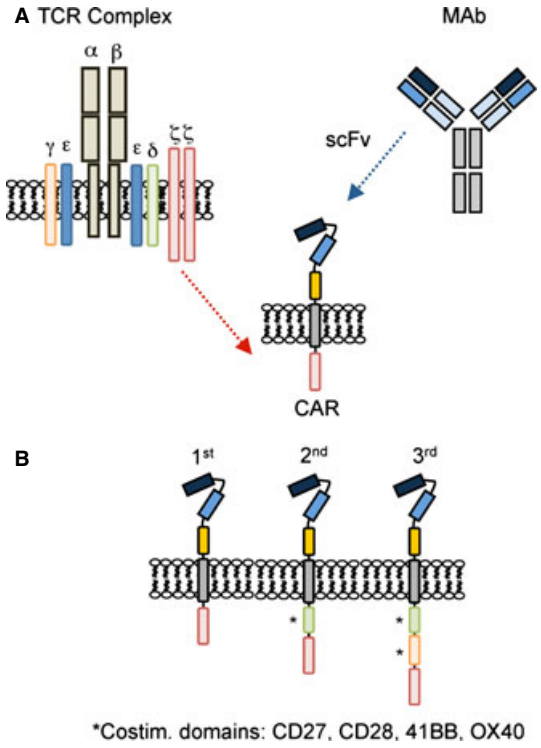

Fig. 1. Chimeric antigen receptor (CAR) design. (A) CARs consist of an ectodomain, commonly derived from a single chain variable fragment (scFv), a transmembrane domain, and an endodomain. (B) Depending on the number of signaling domains, CARs are classified into 1st generation (one), 2nd generation (two), or 3rd generation (three) CARs.

an epitope on CD22 (an antigen expressed by normal and malignant B cells) that was proximal to the B cells' plasma membrane had superior anti-leukemic activity to CAR-T cells that recognized a membrane-distal epitope (10). Antigen binding and subsequent activation can also be modulated by introducing a flexible linker sequence in the CAR, which will also allow expression of two distinct scFvs that can recognize two different antigens (11) (*Figs 1 and 2*). T cells expressing these so-called tandem CARs (TanCARs) may be better able to kill tumor targets expressing low levels of each antigen individually and may also reduce the risk of tumor immune escape due to the emergence of single antigen loss variants.

Because the scFvs used to date in the clinic have almost all consisted of both heavy and light chain-derived antigen binding domains and are often derived from murine monoclonal antibodies, there is considered to be a significant risk of anti-idiotype or anti-mouse antibodies, either of which can block function. Single domain scFvs have therefore also been used to prepare CARs (12). Their smaller ectodomain may render them less immunogenic, although this may come with the cost of lower affinity/specificity. Another

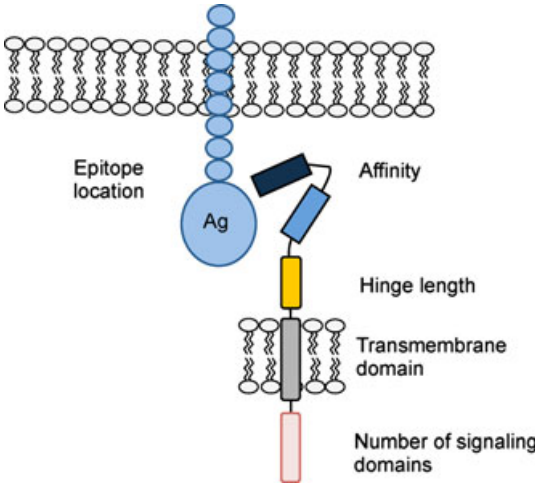

Fig. 2. Critical Chimeric antigen receptor (CAR) components. Optimal CAR activity is determined by epitope location, scFv affinity, hinge and transmembrane domains, and number of signaling domains.

strategy to reduce CAR immunogenicity is to humanize the scFvs, an approach taken for HER2-, EphA2-, and mesothelin-specific CARs (13–15). Unfortunately, this approach does not preclude the development of anti-idiotype antibodies that may be equally inhibitory.

The CAR concept is not confined to using scFvs as the targeting ectodomain, and other ligands and receptors have been substituted. For example, IL13Rα2-specific CARs have been prepared by modifying IL13 molecules to form ectodomains and used clinically (16–18), while NKG2D-ligand and CD70-specific CARs have been constructed by adding a ζ-signaling domain to the cytoplasmic tail of NKG2D or the CD70 receptor (CD27) respectively (19–21). Peptide ligands have also been used as CAR ectodomains. For example, Davies *et al.* (22) designed a CAR containing the promiscuous T1E peptide ligand that will recognize and bind to target cells expressing the ErbB family of receptors. Finally, multiple antigens can be recognized by so called 'universal ectodomains' such as CARs that incorporate an avidin ectodomain to recognize targets that have been incubated with biotinylated monoclonal antibodies (23), or that contain a FITC-specific scFv, which has potent anti-tumor activity in preclinical animal models when given in combination with FITC-labeled monoclonal antibodies (24). These alternative CAR ectodomains have performed well in preclinical studies, but only the IL13Rα2-specific CAR have been tested in humans (25, 26).

Hinge region of CARs

While the ectodomain is critical for CAR specificity, the connecting sequence between the ectodomain to the

transmembrane domain (the hinge region) (*Fig. 1*), can also profoundly affect CAR-T-cell function by producing differences in the length and flexibility of the resulting CAR. For example Guest *et al.* (27) compared the influence of adding a CH2CH3 hinge derived from IgG1 to hingeless CARs specific for carcinoembryonic antigen (CEA), neural small adhesion molecule (NCAM), 5T4, or CD19. While 5T4- and CD19-specific CAR-T cells with a CH2CH3 hinge had enhanced effector function, CEA- and NCAM-specific CAR-T cells had optimal activity without a hinge. More recently, Hudecek *et al.* (7) compared the influence of a CH2-CH3 hinge [229 amino acids (AA)], CH3 hinge (119 AA), and short hinge (12AA) on the effector function of T cells expressing 3rd generation ROR1-specific CARs. They demonstrated that T cells expressing 'short hinge' CARs had superior anti-tumor activity. Other investigators found that a CH2-CH3 hinge impaired epitope recognition of a 1st generation CD30-specific CAR (28). While these results are somewhat conflicting, they all clearly indicate the potential importance of the hinge region in determining outcome of CAR engagement.

At present we do not know the mechanisms underlying the above observational differences, and our dataset is too small for any general predictive rules or algorithm to have emerged as to which hinge will likely best work with which CAR. For the moment, therefore, empiric testing of scFv/hinge domain combinations is required to determine optimal CAR design, although this will likely change as more experimental data and validation studies are available for analysis.

Transmembrane domain of CARs

Between the hinge and the signaling endodomains lies the transmembrane domain. This is usually derived from CD3-ζ, CD4, CD8, or CD28 molecules. Like hinges, the transmembrane domains were initially viewed as inert structural links between the ectodomain and endodomain of the CAR. It is now evident that the transmembrane domain can indeed influence CAR-T-cell effector function. For example, first generation CD19-specfic CAR which contain a CD3-ζ transmembrane domain are less stable over time on the cell surface of T cells in comparison with 2nd generation CD19-specific CAR-T cells with a CD28 transmembrane domain (29). Simply replacing the CD3-ζ transmembrane domain with a CD28 transmembrane domain renders the expression of 1st generation CARs more stable (Dotti *et al.*, unpublished data). Other investigators have shown that an intact CD3-ζ transmembrane domain is essential for measurable signaling by a 1st generation CEA-specific CAR expressed in a T-cell line (30). CARs incorporating a transmembrane domain from native CD3-ζ chain could dimerize and form complexes with endogenous TCRs resulting in enhanced T-cell activation, while CARs containing mutated CD3-ζ transmembrane lacked these interactions.

Endodomain of CARs

Upon antigen recognition, CAR endodomains transmit activation and costimulatory signals to T cells. T-cell activation relies on the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) present in the cytoplasmic CD3-ζ domain of the TCR complex (31). While the majority of current CAR endomains contain an activation domain derived from CD3-ζ, other ITAM-containing domains have been explored including the Fc receptor for IgE-γ domain, which proved to be less effective (32). The selection of costimulatory signaling domains is described in detail in a later section (Provision of co-stimulation to enhance CAR-T-cell activity after antigen-specific receptor engagement).

In conclusion, there is an intricate interplay between scFvs, hinge, transmembrane domain, and endodomain that determines CAR function and there is no single optimal configuration that is 'one size fits all'. For the moment, therefore, CAR receptor optimization remains largely empirical, with testing required in a range of preclinical models.

Antigens suited to CAR-targeted adoptive cellular immunotherapy

Unlike the native TCR, the majority of scFv-based CARs only recognize target antigens expressed on the cell surface, rather than internal antigens that are processed and presented by the cells' MHC. While this limits the detection of a range of tumor specific antigenic epitopes (for example from mutant oncogenes and translocations), CARs have the advantage over the classical TCR that they can recognize structures other than protein epitopes, including carbohydrates and glycolipids. This increases the pool of potential target antigens. Like other cancer immunotherapy approaches, CARs should ideally target antigens that are only expressed on cancer cells or their surrounding stroma (33), such as the splice variant of EGFR (EGFRvIII), which is specific to glioma cells (34). Few human antigens, however, meet this criterion, and the majority of target antigens (*Tables 1 and 2*) are expressed either at low levels on normal

Table 1. **Chimeric antigen receptor (CAR) targets for hematological malignancies**

Antigen	Malignancy	CAR ectodomain, antigen class	Clinical Trial
CD19 (29, 35–44)	B-cell	scFv, protein	Published
CD20 (36, 45–48)	B-cell	scFv, protein	Published
CD22 (10)	B-cell	scFv, protein	
CD30 (49–51)	B-cell	scFv, protein	Ongoing
CD33 (52)	Myeloid	scFv, protein	
CD70 (21)	B-cell/T-cell	Ligand, protein	
CD123 (53)	Myeloid	scFv, protein	
Kappa (54)	B-cell	scFv, protein	Ongoing
Lewis Y (55, 56)	Myeloid	scFv, carbohydrate	Published
NKG2D ligands (20, 57–59)	Myeloid	Ligand, protein	
ROR1 (7)	B-cell	scFv, protein	

cells (e.g. GD2, CAIX, HER2) and/or in a lineage restricted fashion (e.g. CD19, CD20).

Antigens overexpressed by tumor cells

Although the majority of target antigens on tumor cells are shared with normal tissues and are only overexpressed in comparison with normal tissues, many have been targeted by CAR-T cells in preclinical animal models. Unfortunately, these models often cannot accurately predict human toxicities since many of the scFV ectodomains do not recognize non-human counterparts of the targeted antigens, the tissue distribution of which is also frequently species specific. This same limitation, of course, applied to studies with monoclonal antibodies. So to avoid unexpected toxicities, many of the first clinical studies selected target antigens based on the availability of monoclonal antibodies with an acceptable safety profile in humans. Since almost no monoclonal antibody has been free of at least some adverse effects, the decision on whether to proceed with a study in which the CAR is derived from a monoclonal antibody has been determined by careful assessment of the likely risk:benefit ratio for any given target antigen. For example, the EGFR monoclonal antibody cetuximab is FDA approved for the treatment of EGFR-positive cancers, but most patients treated with the monoclonal antibody develop a skin rash due to baseline EGFR expression in epithelial cells (110). Since the overall avidity of EGFR-specific CARs arrayed on a T cell would be

Table 2. **Chimeric antigen receptor (CAR) targets for solid tumors**

Antigen	Malignancy*	CAR ectodomain, antigen class	Clinical Trial
B7H3 (60)	Sarcoma, glioma	scFv, protein	
CAIX (61, 62)	Kidney	scFv, protein	Published
CD44 v6/v7 (63, 64)	Cervical	scFv, protein	
CD171 (65)	Neuroblastoma	scFv, protein	Published
CEA (66)	Colon	scFv, protein	Ongoing
EGFRvIII (67, 68)	Glioma	scFv, protein	Ongoing
EGP2 (69, 70)	Carcinomas	scFv, protein	
EGP40 (71)	Colon	scFv, protein	
EphA2 (14)	Glioma, lung	scFv, protein	
ErbB2(HER2) (72–79)	Breast, lung, prostate, glioma	scFv, protein	Published
ErbB receptor family (22)	Breast, lung, prostate, glioma	Ligand, protein	
ErbB3/4 (80, 81)	Breast, ovarian	scFv, protein	
HLA-A1/MAGE1 (82, 83)	Melanoma	scFV, peptide/protein complex	
HLA-A2/NY-ESO-1 (84)	Sarcoma, melanoma	scFV, peptide/protein complex	
FR-α (85–88)	Ovarian	scFv, protein	Published
FAP† (89)	Cancer associated fibroblasts	scFv, protein	
FAR (90)	Rhabdomyosarcoma	scFv, protein	
GD2 (91–93)	Neuroblastoma, sarcoma, melanoma	scFv, ganglioside	Published
GD3 (94)	Melanoma, lung cancer	scFv, ganglioside	
HMW-MAA (95)	Melanoma	scFv, proteoglycan	
IL11Rα (96)	Osteosarcoma	Ligand, protein	
IL13Rα2 (16–18, 25)	Glioma	Ligand, protein	Ongoing
Lewis Y (55, 97, 98)	Breast/ovarian/pancreatic	scFv, carbohydrate	
Mesothelin (15, 99)	Mesothelioma, breast, pancreas	scFv, protein	Ongoing
Muc1 (100)	Ovarian, breast, prostate	scFv, glycosylated protein	
NCAM (101)	Neuroblastoma, colorectal	scFv, protein	
NKG2D ligands (20, 57–59)	Ovarian, sacoma	Native receptor, protein	
PSCA (102, 103)	Prostate, pancreatic	scFv, protein	
PSMA (104, 105)	Prostate	scFv, protein	
TAG72 (106, 107)	Colon	scFv, carbohydrate	
VEGFR-2† (108, 109)	Tumor vasculature	Ligand/scFv, protein	Ongoing

*Many antigens are expressed on several malignancies; due to space limitations only examples are listed.
†Expressed on the tumor stroma.

greater than the avidity of a bivalent soluble antibody, significant safety concerns were raised about the severity and persistence of skin toxicity that have so far precluded clinical trials of EGFR-specific CAR-T cells by systemic administration. Conversely, GD2- and HER2-specific monoclonal antibodies have a favorable safety profile, which led to testing of CAR-T cells in humans (73, 91, 92, 111).

Lineage-specific antigens

Lineage-specific targets have been mainly explored for hematological malignancies (29, 37, 40, 42–44, 112). For example, CAR-T-cell treatment of B-cell malignancies can be used to target a highly and consistently expressed lineage-specific antigen (e.g. CD19, CD20), even though it is also expressed by normal B cells, since replacement therapy using intravenous immune globulin is feasible. In general, however, it might be preferable to target more lineage-associated antigens. For example, in many B-cell malignancies it is possible to target either the κ- or λ-light chain, since normal B cells express one or other of these antigens, while all the cells of the (clonal) malignancy will express a single light chain (54). In addition, as discussed below (Increasing the safety of CAR-T cells), the increasing potency of later generation CARs and their combination with other strategies to improve their function will almost inevitably increase their potential for 'on-target antigen but off target tissue' activity, thereby producing unexpected toxicities even when targeting lineage specific antigens, since these might be aberrantly expressed at low levels elsewhere.

Other considerations for antigen selection

Targeting single antigens carries the inherent risk of immune escape (113–115), which can be reduced by targeting multiple antigens. In preclinical studies, targeting HER2 and IL13Rα2 with CAR-T cells resulted in enhanced anti-tumor effects in comparison with CAR-T cells that targeted a single antigen (116). As discussed below (Increasing the safety of CAR-T cells), expressing multiple CARs in T cells also has the potential to increase safety by generating T cells that recognize a unique antigen pattern that is only present on tumor cells. Targeting antigens exclusively expressed by cancer cells does not, however, address the fundamental problem of selecting variants that lack expression of the target antigen. Such escape variants are common because of the marked genetic instability of most cancer cells (117). One solution is to target antigens expressed on the tumor stroma, a component that is critical for tumor growth and is more genetically stable. As discussed below (Targeting the cellular components of tumor stroma) investigators have therefore targeted fibroblast activation protein (FAP) expressed on cancer associated fibroblasts (CAFs) or vascular endothelial growth factor receptor (VEGFR)-2 expressed on the endothelial cells of the tumor vasculature (89, 108).

Initial clinical experience with CAR-T cells

Initial studies in humans were conducted with T cells expressing the 1st generation CARs illustrated in *Fig. 1*. Investigators used CAR-T cells to target hematological malignancies as well as solid tumors. Even when these CAR-T cells were combined with lymphodepletion (to reduce Treg mediated inhibition and favor homeostatic expansion of the infused cells), the results were uniformly disappointing, with minimal expansion or persistence *in vivo*, and with no unequivocal evidence of anti-tumor activity. For example, two patients received CD20-specific CAR-T cells post autologous stem cell transplant, and four patients received CD19-specific CAR-T cells in combination with IL-2 outside the transplant setting (36, 45). While high doses of T cells were administered (up to 2×10^9 cells/m^2), T-cell persistence was less than 1 week. Despite the short persistence, two patients developed antibodies directed to the CAR. The clinical efficacy of these CAR-T cells was difficult to assess since patients either received T cells post transplant or received additional therapies post T-cell infusion.

First generation CAR-T cells targeting carbonic anhydrase IX (CAIX), CD171, folate receptor α (FR-α), and GD2 were evaluated in patients with advanced stage solid tumors (61, 87, 91, 92, 118). Lamers *et al.* (61) infused renal cell carcinoma patients with polyclonal T cells expressing a 1st generation CAIX-specific CAR. Two of the first three patients developed hepatitis due to CAIX expression on bile ducts. Both patients also developed potent anti-CAR immune responses resulting in limited T-cell persistence (61). Subsequently, pretreatment with CAIX monoclonal antibodies of four patients prior to CAR-T-cell transfer prevented hepatitis and abrogated the induction of anti-CAR immune responses (118). While this resulted in prolonged T-cell persistence, no clinical benefit was observed. Six neuroblastoma patients received up to $10^9/m^2$ of $CD8^+$ T-cell clones expressing 1st generation CARs specific for CD171 (65). Infusions were well-tolerated, but T cells persisted only for 6 weeks, and only 1 of 6 patients had a partial response 8 weeks post

T-cell infusion. Kershaw *et al.* (87) evaluated the safety and efficacy of 1st generation FR-α CAR-T cells in patients with ovarian cancer. Eight patients received up to 5×10^{10} CAR-T cells in combination with IL-2, whereas six patients received CAR-T cells in combination with an allogeneic PBMC vaccine. T cells persisted less than 3 weeks in all but one patient and did not specifically home to tumor sites as judged by ^{111}Indium scintigraphy. No anti-tumor activity was observed. Because of these disappointing outcomes, extensive efforts have been made to enhance the effector function of CAR-T cells *in vivo*, which are discussed in detail in the following sections.

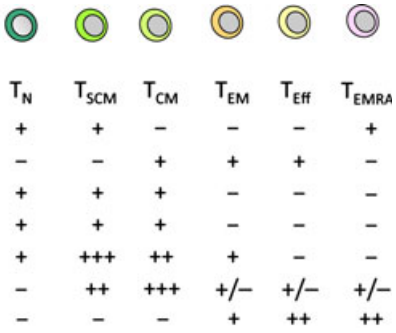

Fig. 3. T-cell subsets. The phenotypic properties of T-cell subsets. − indicates absence and + presence of the specific cell surface marker. Functional properties for each subset are also shown. − indicates lack of the functional property; +, ++, +++ indicates the degree (low, intermediate, high) of the specific property.

Optimal T-cell subset in which to express CARs

The ideal T-cell target in which to graft CAR molecules should be the subset that can traffic to tumor sites, receive appropriate co-stimulation, and retain a profile predictive of prolonged *in vivo* survival. Moreover, the T-cell chosen should preferably not be able to produce toxicity *in vivo* due to the inappropriate activation of signaling pathways in otherwise quiescent T-cell subtypes, or because of disruption to the otherwise tightly coordinated activation and subsequent contraction processes of T cells that are dependent on both antigenic stimulation and physiological costimulation and inhibition.

Identification of optimal T-cell surface phenotype

A number of investigators have tried to identify the phenotypic profile of the optimal subset to allow this component of the T-cell system to be separated and selectively transduced, and produce maximum benefit with minimum adverse effects (119, 120) (*Fig.* 3). These efforts are based on evidence that signals for memory T-cell development are received during the initial expansion of T cells and that the pattern of development and cell fate are influenced by the stimuli received during initial exposure to antigen (priming). The affinity of the TCR engagement, the balance of costimulatory versus inhibitory cellular and soluble signals, as well as other environmental cues (121, 122) all dictate the diversity of T-cell subsets which generate memory T cells and effector T cells (Teff) either linearly, progressively, or through asymmetric division (123).

The obvious population in which to express CARs is the Teff, since this subset are potent anti-tumor effector cells (124). Teff cells are, however, less appealing for adoptive immunotherapy because of their limited proliferative capacity and persistence *in vivo*. Investigators instead are now concentrating on memory T-cell subsets, which are traditionally divided into central and effector memory cells (Tcm and Tem) based upon the expression of CD62L and CCR7 (maintained on Tcm and lost on Tem) (125, 126). These cells are 'antigen-experienced', can expand substantially *in vivo* and are long-lived. Many studies have now shown that a balanced composition of both Tcm and Tem subsets can effectively and rapidly control repeated exposure to pathogens over a prolonged period (127). Because of these characteristics, Tcm and Tem cells have been considered ideal vehicles for grafting CARs. Since CD62L expression can be used to identify T cells with memory characteristics, investigators have used positively selected CD62L cells to express a CAR and shown superior activity in a mouse model (128). A clinical study based on this approach has been opened at the Fred Hutchinson Cancer Research Center.

More recently, a phenotypically defined T-cell subset with true stemness properties (the Tscm) has been identified (129). These cells have enhanced proliferative potential and survival *in vivo* and can differentiate into memory and effector populations. If they are indeed a truly distinct subset in humans, they may allow investigators to identify a T-cell subset suited to genetic manipulation that will also be best able to mediate complete and durable remission in cancer patients.

Functional T-cell selection

An alternative approach to using surface-phenotype based selection of T-cell subsets prior to CAR expression is to employ T cells already selected *in vivo* for their established capacity to act as Teff, to enter the memory pool, and to

re-expand on re-exposure to antigens. Virus-specific cytotoxic T lymphocytes (CTLs) are an example of such cells, and in addition to their potential for life-long persistence, virus-specific CTLs contain both $CD8^+$ and $CD4^+$ subsets, with the latter compartment critical for long-term persistence of the former (130, 131). Virus-specific CTLs are also well-characterized for expression of homing/chemokines receptors commensurate with their capacity for trafficking to and residing in the designated lymphoid or non-lymphoid tissue (132). Their potential value as CAR-expressing effector cells is considered below.

Provision of costimulation to enhance CAR-T-cell activity after antigen-specific receptor engagement

T-cell costimulation is mediated by a multiplicity of receptor-ligand interactions, and plays a fundamental role in preventing the induction of anergy in T lymphocytes upon engagement with the target antigen (121). Tumor cells and the tumor microenvironment are deficient in costimulation and favor the induction of T-cell anergy due to their lack of expression of costimulatory molecules such as CD80 and CD86 that are ligands for the CD28 receptor expressed by activated effector T cells (133). Adoptively transferred T lymphocytes engineered to express CARs are not immune from tumor-induced anergy. In the absence of CD80/CD86 co-stimulation, engagement of antigen through the CAR produces T-cell hyporesponsiveness (134). In addition, when tumor-specific T cells are expanded *ex vivo* they may lose expression of CD28, particularly if culture is prolonged, and thus become unresponsive to tumor cells or bystander cells expressing costimulatory molecules.

CAR molecules can be engineered to overcome the lack of co-stimulation by tumor cells. Endodomains derived from well-characterized co-stimulatory molecules such as CD28 (54, 104), CD134/OX40 (134), CD137/4-1BB (135, 136), and CD27 (137) can be incorporated within CARs to provide direct T-cell co-stimulation after CAR antigen binding (*Fig. 1*). Since each of these co-stimulatory molecules activates different signaling pathways [e.g. phosphatidylinositol 3-kinase (PI3K)] for CD28 versus tumor necrosis family (TNF)-receptor-associated factor adapter proteins for 4-1BB and OX40), multiple endodomains can be included in a single CAR and thereby recruit multiple signaling pathways, potentially maximizing the costimulatory benefits (134, 136). Although the mechanism of the effector cell immunological synapse formation by CAR molecules has not been elucidated, it is likely that CAR cross-linking upon antigen-binding leads to the physical recruitment and dimer formation of costimulatory endodomains incorporated within the CAR and consequent activation of the downstream signaling pathways. An alternative means of providing costimulation that may occur independently of CAR cross-linking relies on the trans- and auto-co-stimulation achieved when CAR-T cells are further engineered to express either CD80 or 4-1BBL molecules that are separated from CAR molecules, but this approach has not yet been clinically tested (138).

Identification of optimal endodomains

That incorporation of costimulatory endodomains in CARs enhances the proliferation and activation of CAR-T cells in humans *in vivo* has been clearly demonstrated by direct comparison of the fate of CAR-T cells with and without an included co-stimulatory endodomain (29). Efforts to predict in advance which endodomains or combination of endodomains will prove to be optimal in humans has been much more problematic. *In vitro* experiments and xenogeneic mouse models have been used to dissect and compare the effects of incorporating each costimulatory endodomain into CARs, and it has become evident that the results are often contradictory and may not predict events in clinical studies. For example, when CD28, 4-1BB or combinations of multiple costimulatory endodomains were compared, the relative potency of each was dependent on the target antigen, the biology of the tumor cells and the mouse strains used (139, 140). Ultimately, identification of the optimum choice of costimulatory endodomains(s) for a given CAR and tumor cell target may have to be resolved in clinical trials.

Direct comparison of costimulatory endodomain activity in humans

One means of directly determining the value of each specific CAR costimulatory moiety in patients is to simultaneously infuse each patient with two or more T-cell products, each expressing CARs with identical specificity and sequence, but with different costimulatory components. Using this approach, we infused six lymphoma patients simultaneously with two T-cell products expressing the same CD19-specific CAR but encoding either the CD3-ζ chain of the TCR alone or both the CD3-ζ chain and the CD28 endodomain. We demonstrated that CD28 costimulation within the CAR indeed promotes superior *in vivo* expansion of CD19-specific CAR-T cells (29). In a similar current study at Memorial Sloan-Kettering and University of Pennsylvania, investigators are comparing the simultaneous infusion of T cells expressing CD19-specific CARs incorporating either the

CD28 or 4-1BB endodomains. We will initiate a new study to compare CD19-specific CARs incorporating either CD28 alone or the combination of CD28/4-1BB endodomains. Although impressive results have already been reported in B-cell malignancies incorporating the 4-1BB endodomain within a CD19-specific CAR (41, 42, 44), these direct comparator studies retain fundamental importance if we are to know definitively how CAR-mediated T-cell costimulation affects T-cell fate and anti-tumor activity, since this dual component study design avoids the inevitable and significant confounding variables associated with small phase I studies enrolling heterogeneous patients with heterogeneous disease.

Role of lymphodepletion

The expansion, persistence, and anti-tumor activity of CAR-T cells may be enhanced if the cells are given after administration of lymphodepleting drugs (141). These benefits may result from reduction of Treg in the lymphoid tissues and in tumor and from the production of cytokines such as IL-7 and IL-15 that may favor expansion of infused cells. At present, however, there is no unequivocal evidence that lymphodepletion benefits the outcome, and the potential toxicities may ultimately negate the putative benefits (38, 73).

Clinical experience with 2nd and 3rd generation CAR-T cells: hematological malignancies

The most impressive clinical results with CAR-T cells so far has been achieved with polyclonal T cells expressing CD19-specific CARs either with CD28.ζ or 41BB.ζ signaling domains (37, 40–44). These studies have been recently reviewed in detail elsewhere (142). Complete responses were observed post infusion of 2nd generation CAR-T cells in patients with $CD19^+$ hematological malignancies including non Hodgkin's lymphoma (NHL), chronic lymphocytic leukemia and acute lymphoblastic leukemia. Anti-tumor activity was dependent on significant T-cell expansion *in vivo*, which was associated in several patients with a life-threatening cytokine storm (42, 44). The clinical picture was reminiscent of hemophagocytic syndrome and patients responded either to a combination of steroids, TNF-α antibody (infliximab), and IL-6 receptor antibody (tocilizumab) or to monotherapy with tocilizumab (42, 44). While addition of co-stimulatory domains dramatically increased the expansion and persistence of polyclonal T cells, this benefit was not observed with T-cell clones. Three patients received T-cell clones expressing 3rd generation CD20-specific CARs after lymphodepletion with cytoxan (143). T-cell persistence was limited; two patients with no evaluable disease remained disease free for 12 and 24 months, and a 3rd patient had a partial response that lasted for 12 months. This study indicates that T-cell clones, regardless of the endodomain used, can have limited T-cell function *in vivo*.

Besides targeting hematological malignancies with CD19 and CD20 CAR T cells, we are currently conducting clinical studies with polyclonal T cells expressing 2nd generation CARs specific for the κ-light chain of human immunoglobulin (see 'Control of toxicities') or for CD30. We have generated CARs specific to selectively target κ^+ lymphoma/leukemia cells, while sparing the normal B cells expressing the non-targeted λ-light chain, thus minimizing the impairment of humoral immunity associated with the depletion of all normal B lymphocytes (54). This approach is now in clinical trial using a 2nd generation CAR encoding the CD28 endodomain and clinical responses including CRs have been observed (144). We have also implemented clinical trials using a 2nd generation CAR specific for the CD30 antigen (50), which is present on most Hodgkin's lymphoma (HL) and some NHL cells. The generation of functional CAR-T cells has been successful in the patients with refractory/relapsed diseases, of whom four have been treated so far without toxicities.

In contrast with B-cell malignancies, limited clinical experience is available for CARs redirected to T-cell or myeloid-derived malignancies. While preclinical studies have demonstrated the potent anti-acute myeloid leukemia (AML) effects of CD123-specific or Lewis-Y antigen-specific CAR T cells (53, 55), only one clinical study with Lewis-Y antigen-specific CAR T cells is in progress for AML patients (56).

Solid tumors

Limited clinical experience is currently available with targeting solid tumor antigens using 2nd or 3rd generation CARs. One patient who received 10^{10} T cells expressing a 3rd generation HER2-specific CAR and a lymphodepleting chemotherapy regimen consisting of cytoxan and fludarabine, in combination with IL-2 rapidly developed acute respiratory distress syndrome and died (73). We have infused up to $10^8/m^2$ T cells expressing a 2nd generation HER2-specific CAR to patients with osteosarcoma. While no overt toxicities were observed, the anti-tumor activity has so far been limited (111). Clinical studies with T cells expressing a 3rd generation EGFRvIII-specific CAR for glioma patients are in

progress, and another study has treated patients with pancreatic cancer with a 2nd generation mesothelin-specific CAR, one of whom developed an anaphylactic reaction (145).

Using physiological costimulation through the native T-cell receptor

The costimulation provided by second or later generation CARs is non-physiological, since it does not occur in the same regulated tempero-spatial sequence that follows a T-cell encounter with antigen on professional antigen-presenting cells. Indeed, the overabundance of stimulation from a 2nd or 3rd generation CAR may be toxic to the T-cell itself (for example by activation induced cell death) or to the recipient of the T cells (for example by the rapid production of pro-inflammatory cytokines). An alternative approach outlined in the section 'Optimal T-cell subset in which to express CARs' relies instead on restricting expression of CARs to a specific T-cell subset such as virus-specific CTLs for which physiologic CD80/CD86 costimulation is provided by professional antigen-presenting cells when viral latent antigens processed by these APCs are encountered by CTLs through their native TCRs (35, 50, 146). While attractive in principle, the choice of suitable viral antigen specificity of a CTL is limited, as the virus associated antigens need to be encountered relatively frequently and in the presence of a broad array of costimulatory molecules. Epstein Barr virus (EBV)-infected cells may be a valuable resource for this purpose. B lymphocytes expressing EBV-antigens persist lifelong in seropositive individuals and boost both MHC class-I and class-II EBV-specific CTLs targeting latent antigens (132). Importantly, *ex vivo* expanded EBV-specific CTLs retain the same property of longevity as circulating cells and persist long-term after adoptive transfer (130, 131). Based on this evidence, we demonstrated preclinically (50, 146) and then clinically that EBV-specific CTLs engrafted with CARs produce anti-tumor effects against tumors targeted by the CAR, while retaining their physiological costimulation through their native antigen-specific αβTCRs (91, 92).

Clinical experience with CAR-engrafted virus-specific CTLs

Our group expressed a 1st generation CAR directed to GD2 on EBV-specific CTLs and gave them to 11 children with advanced neuroblastoma (91, 92). By comparing EBV-specific CTLs and activated T cells expressing the same but distinguishable 1st generation CAR, we found that CAR-expressing EBV-specific CTLs initially persisted in the circulation at a higher level and longer than activated T cells and that 5 of the 11 patients with active disease showed tumor responses or necrosis. Three of them had complete responses (sustained in two), while an additional two with bulky tumors showed substantial tumor necrosis. Nevertheless, neither of the CAR-T cell populations expanded *in vivo*, and patients with massive tumor burdens were little helped. Subsequent GD2-CAR studies are using lymphodepletion in combination with second and third generation vectors and are described in the next sections.

We next designed a study in which adult patients with B-cell malignancies were infused simultaneously with EBV-specific CTLs engrafted with a 1st generation CD19-specific CAR and activated T cells expressing a 2nd generation CD19-specific CAR encoding the CD28 endodomains. Major limits, however, quickly emerged for this study. EBV-specific CTLs could be manufactured only in a minority of potential referrals, since the majority of the lymphoma patients had received anti-CD20 antibody (rituximab) as part of their standard of care, so eliminating B cells and precluding generation of the EBV+ B-lymphoblastoid cell lines required the *ex vivo* expansion of CTLs. Both products were available for five patients, but CD19-specific CAR-expressing EBV-specific CTLs did not persist longer than activated T cells expressing the 2nd generation CAR, likely because EBV-infected B cells in these patients were lacking (authors, manuscript in preparation). Recognizing that EBV-expressing target cells will not be available to use as stimulators in lymphoma patients, we broadened the virus specificity of our cells to include cytomegalovirus (CMV) that also has chronic persistence. This clinical trial has been opened for allogeneic stem cell transplant recipients (147). Similarly, a trial using CTLs specific for three viruses, adenovirus, EBV and CMV, engrafted with a GD2-specific CAR has been recently initiated in the post allogeneic transplant setting in patients with refractory/relapsed neuroblastoma (148). We are also exploring the use of CMV-specific T cells that are genetically modified with a 2nd generation HER2-specific CAR for the adoptive immunotherapy of glioblastoma. Since CMV antigens are present in latently infected leukocytes and are also detected in the tumor itself (149–152), this approach may enhance *in vivo* persistence and expansion of adoptively transferred T cells and also directly increase the anti-tumor activity of transferred T cells.

Beyond costimulation: other approaches to improving expansion, persistence, and function of CAR-T cells

The clinical studies described above confirm the preclinical data showing that introducing costimulation for CARs is necessary but insufficient *per se* to reverse all the immune inhibitory mechanisms of cancer. This is particularly apparent when solid tumors rather than hematologic malignancies are being targeted. Solid tumors and their microenvironment lack the conventional costimulatory molecules that may be present on (for example) malignant and normal B lymphocyte targets and have developed intricate systems to suppress the immune system (133, 153, 154) (Fig. 4). Thus, malignant cells and their supporting stroma secrete immunosuppressive cytokines such as transforming growth factor-β (TGFβ) or IL-10, attract immunosuppressive cells such Tregs or myeloid derived dendritic cells (MSDCs), inhibit dendritic cell maturation, express molecules on the cell surface that suppress immune cells including FAS-L and PD-L1, and create a metabolic environment (e.g. high lactate, low tryptophan) that is immunosuppressive.

While T-cell costimulation mediated by CD28 and 4-1BB endodomains in CAR molecules may overcome some of the above inhibitory effects, other causes of T-cell anergy are more resistant. Additional engineering of CAR molecules has therefore been exploited as countermeasures to the inhibitory mechanisms developed by tumor cells and their microenvironment and to further enhance CAR-T-cell activity.

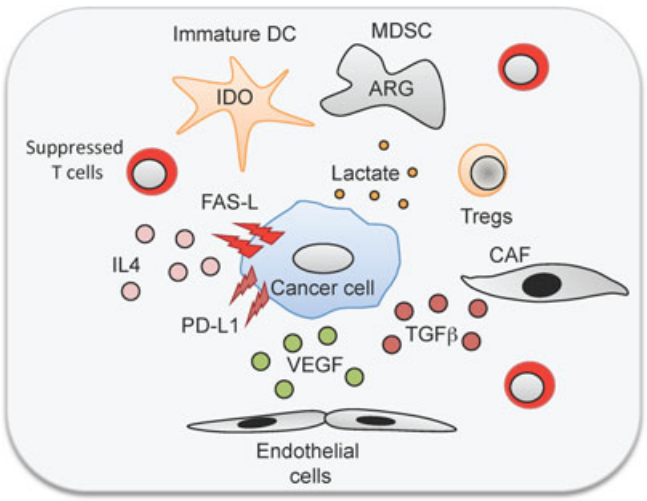

Fig. 4. Immune evasion strategies of tumors. Malignant cells and their supporting stroma (i) secrete immunosuppressive cytokines such as transforming growth factor-β (TGFβ) or interleukin-10 (IL-10); (ii) attract immunosuppressive cells such Tregs or myeloid derived dendritic cells; (iii) inhibit dendritic cell maturation; (iv) express molecules on the cell surface that suppress immune cells including FAS-L and PD-L1, and; (v) create a metabolic environment (e.g. high lactate, low tryptophan) that is immunosuppressive.

Three broad approaches have been adopted as countermeasures to overcome tumor immunosuppression: (i) increasing the level of CAR-T cell activation or decreasing physiological downregulation; (ii) engineering the CAR-T cells to be resistant to the immune evasion strategies used by the tumor; or (iii) targeting the cellular components of tumor stroma (Fig. 5). Any one countermeasure may affect more than one mechanism of tumor immunosuppression.

Addition of immunostimulatory cytokines/cytokine receptor genes

IL-2 is released by 2nd generation CAR-T cells following receptor engagement, but is not by itself sufficient to reverse T-cell anergy (155, 156). Moreover, this cytokine may increase the number and activity of local Tregs (157). Cytokines that are not directly produced by T cells upon antigen stimulation but are biologically active in T cells may be better placed to accomplish the goal of selective CAR-T-cell activation and reversal of anergy without Treg recruitment.

Of these cytokines, IL-15 is perhaps the most promising. IL-15 is mainly produced by monocytes/macrophages and dendritic cells. It shares a common γ-chain with IL-2 and IL-7 but also requires a private chain in the receptor, IL-15Rα, that is expressed on antigen-presenting cells, monocytes, and macrophages (158). IL-15 selectively stimulates these target cells through a cross-presentation mechanism (159). IL-15 promotes the proliferation of T lymphocytes and also prevents apoptosis and exhaustion (155, 160), reverses anergy (155), stimulates long-lasting antigen-experienced memory cells (161), and overcomes Treg-mediated inhibition (156, 162–164). IL-15 can be used either as a growth factor for the *ex vivo* expansion of CAR-T cells, where it may 'imprint' long-lasting resistance to Tregs (39, 165), or as a recombinant protein *in vivo* to support T-cell expansion after adoptive transfer (165), thereby enhancing the anti-tumor activity of adoptively transferred T cells in animal models. Preliminary clinical studies showed that systemic administration of recombinant IL-15 may be highly toxic, and the cytokine may be better tolerated if production is confined to the tumor location. We have described how CAR-T cells can be genetically modified to produce their own IL-15 and achieve the hoped-for benefits at the tumor site while avoiding systemic toxicity (156, 163). Locally produced IL-15 improves CAR-T-cell expansion and persistence *in vivo* and renders CAR-T cells resistant to the inhibitory effects of Tregs by activation of the PI3K pathway

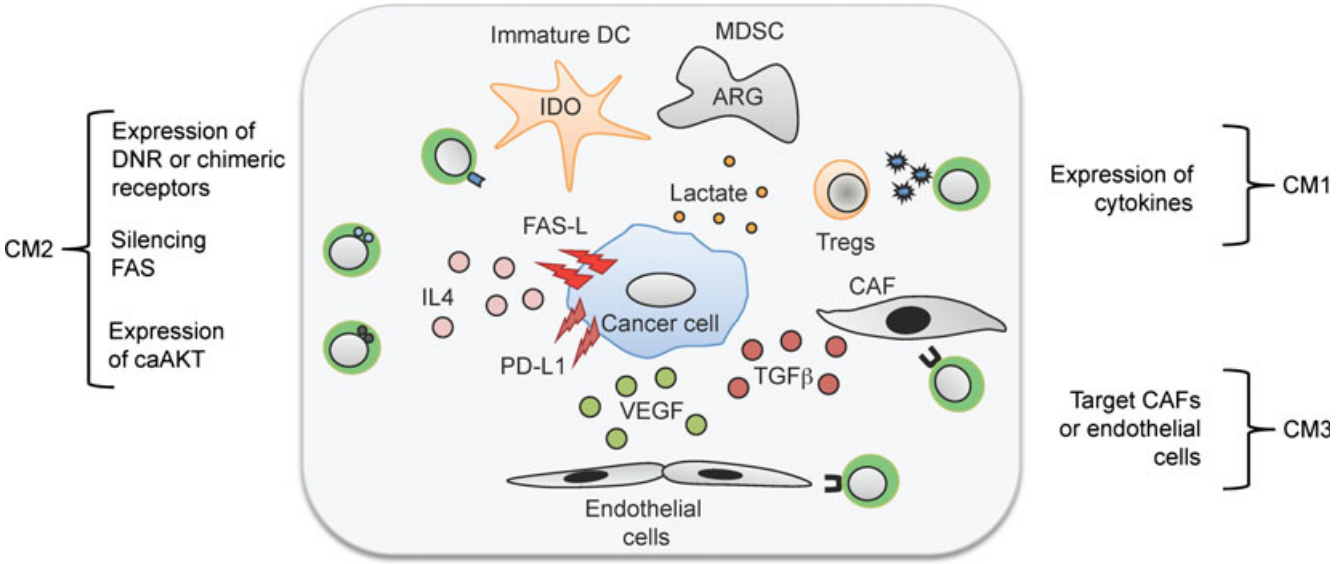

Fig. 5. Overcoming tumor-mediated immunosuppression. Countermeasures can be divided into the following strategies: (i) increasing the level of CAR-T-cell activation or decreasing physiological downregulation; (ii) engineering the CAR-T cells to be resistant to the immune evasion strategies used by the tumor; and (iii) targeting the cellular components of tumor stroma.

resulting in increased expression of anti-apoptotic molecules such BCL-2 (CM2). We plan to test this approach in a clinical trial.

Local production of other cytokines such as IL-7 and IL-12 may also be beneficial. IL-7 shares the γ-chain of its receptor with IL-2 and IL-15 and plays a crucial role in maintaining the homeostasis of mature T cells and the maintenance of memory T cells (166). Clinical studies have shown a good safety profile compared with the other γ-chain cytokines. Since systemic administration of recombinant IL-7 is well-tolerated (167), we and other investigators are manipulating the IL-7/IL-7Rα signaling axis in antigen-specific T cells to selectively promote a robust and selective expansion of these cells following IL-7 exposure (168). Although this approach has not yet reached clinical trial, studies of a third cytokine, IL-12, are more advanced. IL-12 is a pro-inflammatory cytokine that promotes Th1 differentiation and links innate and adaptive immunity (169). Like IL-15, the transgenic expression of IL-12 in tumor-specific T cells significantly increases their anti-tumor activity (170, 171) by directly enhancing T-cell activity and by increasing their production of Th1 cytokines (172). In addition, IL-12 may help to reverse the immunosuppressive tumor environment by triggering the apoptosis of inhibitory tumor-infiltrating macrophages, dendritic cells, and MSDCs through a FAS-dependent pathway (173). While there are safety concerns with regards to constitutive IL-12 expression, there are several mechanisms available to restrict IL-12 production to activated T cells at tumor site by using inducible expression systems (171). Transgenic IL-12 expression by tumor-infiltrating T lymphocytes is currently being tested in a clinical trial at the National Cancer Institute.

Ultimately, it may be possible to bypass the use of cytokines/cytokine receptors and to directly manipulate T-cell pathways that influence cell growth and survival. For example, expression of a constitutively active form of serine/threonine AKT (caAKT), which is a major component of the PI3K pathway, improves T-cell function and survival, since caAKT-expressing T cells have sustained higher levels of NF-κB and of anti-apoptotic genes such as Bcl-2, resulting in resistance to tumor inhibitory mechanisms (174). This approach is at an early stage and may lack the selectivity necessary for safe use.

Blocking the immune check points

Instead of adding stimulatory signals (costimulation, cytokines/cytokine receptors), the function of CAR-T cells may be enhanced by blocking downregulatory signals. Antibodies that block the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) or the programmed death-1 (PD-1) receptor or the PD-L1 ligand have produced encouraging clinical results as single agents (175, 176). CTLA-4 is expressed by activated T cells and acts as a co-inhibitory molecule of T cells after engaging CD80 and CD86 expressed by antigen-presenting cells (177). PD-1 also acts as an inhibitory receptor for T cells by engaging the PD-L1 counter-receptor, expressed by antigen-presenting cells in a regulated manner and constitutively by many tumors (178). Antibodies that block the CTLA-4/CD80/CD86 or PD-1/PD-L1 axes

therefore prevent the physiological contraction of the T-cell immune system. The CTLA-4 antibody ipilimumab has shown remarkable effects in a randomized clinical study in a subset of melanoma patients (175) and promising results have also been reported for antibodies blocking PD-1 or PD-L1 in patients with renal or pulmonary carcinoma (176). The combination of these antibodies with adoptive transfer of CAR-T cells is a logical evolution of the current clinical protocols to prolong the effector function of CAR-T cells at sites of solid tumor. This combination does, however, have the potential for uncontrolled proliferation and activation of the CAR-T cells (179) and will need to be examined using careful dose escalation studies and – ideally – with other safety interventions in place (see 'Increasing the safety of CAR-T cells').

Instead of influencing receptors for inhibitory signals, it is also possible to directly silence genes that render T cells susceptible to inhibitory signals in the tumor microenvironment. For example, many tumor cells express FAS ligand (Fas-L), and silencing FAS expression in T cells prevents FAS-induced apoptosis (180).

Dominant negative and chimeric cytokine receptors

CAR-T cells can be engineered to be resistant to cytokines such as IL-4 and TGFβ that are widely used by tumors as an immune evasion strategy (*Figs 4 and 5*). TGFβ promotes tumor growth and limits effector T-cell function through SMAD-mediated pathways resulting in decreased expression of cytolytic gene products such as perforin, decreased cell proliferation, and increased apoptosis (181, 182). These detrimental effects can be negated by modifying T cells to express a dominant-negative TGFβ receptor type II (TGFβ-DNR), which lacks most of the cytoplasmic component including the kinase domain (183, 184). DNR expression interferes with TGFβ-signaling, thereby blocking TGFβ-induced SMAD2 phosphorylation so that T-cell effector function is sustained even in the presence of TGFβ. We have used the TGFβ-DNR to modify T cells directed to tumors through their native TCRs. We then gave these TGFβ-resistant T cells specific for the EBV antigens LMP1/LMP2 into patients with EBV-positive HL and NHL and have obtained clinical benefit including complete responses (185) even in patients who failed with LMP1/LMP2-specific T cells expressing only wildtype TGFβ receptor type II. Other clinical studies are now in progress in which the TGFβ-DNR is expressed in CAR-T cells.

It is also possible to engineer T cells to actively benefit from the inhibitory signals generated by the tumor environment, by converting inhibitory into stimulatory signals. For example, linking the extracellular domain of the TGFβ RII to the endodomain of Toll-like receptor 4 results in a chimeric receptor that not only renders T cells resistant to TGFβ, but also induces T-cell activation and expansion (186). Chimeric IL-4 receptors are another example of these 'signal converters'. Many tumors secrete IL-4 to create a Th2-polarized environment, and two groups of investigators have shown that expression of chimeric IL-4 receptors consisting of the ectodomain of the IL-4 receptor and the endodomain of the IL-7Rα (187) or the IL-2Rβ chain (188) enable T cells to proliferate in the presence of IL-4 and retain their effector function including Th1-polarization.

Targeting the cellular components of tumor stroma

Most solid tumors have a stromal compartment that supports tumor growth directly through paracrine secretion of cytokines, growth factors, and provision of nutrients and that also contributes to tumor-induced immunosuppression (189–192). This compartment may be a suitable target for CAR-T cell therapy. For example, we have shown in preclinical studies that T cells expressing CARs specific for FAP expressed on CAFs have potent anti-tumor effects, which is enhanced when they were combined with tumor-specific CAR-T cells (89). Hence, targeting CAFs has the potential to improve the anti-tumor activity of adoptively transferred CAR T cells. Other investigators are targeting the tumor vasculature with CARs (108, 109). These studies initially targeted VEGFR-2 using a VEGF-based CAR, but more recent preclinical studies have used a VEGFR-2-specific scFv (108). The combination of CAR-T cells targeting vasculature and tumor cells was again more effective than CAR-T cells targeting either component alone. In addition combining VEGFR-2-specific CARs and IL-12 in T cells was sufficient to eradicate tumors, indicating another means of potentiating effects (193). Any approach that targets antigens present on normal tissue has to consider the inevitable safety concerns, but while these require consideration, clinical studies evaluating the safety and efficacy of the approach, for example with VEGFR-2-specific CAR-T cells, are in progress.

Increasing the safety of CAR-T cells

The efficacy of CAR-modified T cells has not been devoid of toxicities. These fall into four categories.

Toxicity from the gene delivery system

Up to now the most effective approaches to express CARs in human T lymphocytes have been based on γ-retroviral

vectors and lentiviral vectors (41, 42, 44, 194). Both vectors allow robust and stable expression of CARs in T cells without requiring complex and long procedures of *ex vivo* selection. Genomic integration of viral vectors may, however, cause toxicities due to insertional mutagenesis. Unlike past experience with γ-retroviral vector-mediated gene transfer to $CD34^+$ hematopoietic progenitor cells (195), insertional mutagenesis leading to lymphoproliferation of T lymphocytes (including CAR-T cells) has not yet occurred, perhaps because integration is occurring into more differentiated cells with fewer developmental pathways open to disruption by integration events.

On target toxicities

This second type of toxicity is directly attributable to T cells engaging the targeted antigen. For example, the infusion of long-term persisting CD19-specific CAR-T cells is followed by long-term elimination of all cells bearing the CD19 antigen, irrespective of whether they are malignant or normal and leads to profound and prolonged B-cell aplasia and ultimately hypogammaglobulinemia (37, 41–44). This particular toxicity may be ameliorated by infusing immunoglobulin preparations.

On biological target but off organ toxicities

Other on-target toxicities may be more severe and less amenable to correction, particularly if they are on target but 'out of organ'. For example, targeting the carbonic anhydrase IX that is highly expressed by renal cell carcinoma also causes significant liver toxicity, since the targeted antigen is also expressed by cells of the biliary tree (61, 118). Similarly, a fatal adverse event occurred in a patient infused with HER2-specific CAR-T cells which may have been due to low level HER2 expression in the pulmonary parenchyma or vasculature (73).

Systemic inflammatory response syndrome (SIRS) or cytokine storm

This toxicity is attributable to rapid and extensive activation of infused CAR-T cells upon antigen engagement, with general perturbation of the immune system, and the associated release of high levels of proinflammatory cytokines, such as TNF-α and IL-6 (42, 44). This toxicity has now been observed after administration of CAR-T cells with several different specificities and, while often associated with a tumor response and potentially reversible, it remains a major concern for broader introduction of the approach.

Controlling toxicity from the gene delivery system

Although oncogenicity from retroviruses is currently only a hypothetical concern for CAR-T cells, there is considerable interest in developing alternative vector systems that retain significant genomic integration capacity, but are based on DNA plasmids such as the transposon/transposes system which may be less likely to selectively integrate in critical sites in the genome (196, 197). A phase I study in which T cells are engineered to express a CD19-specific CAR encoded in a Sleeping Beauty transposon system has been recently approved by the FDA and the clinical trial is ongoing at MD Anderson Cancer Center.

Controlling on target toxicity

To prevent on target toxicity accurate antigen selection, and careful dose escalation of CAR-T cells remain fundamental of CAR-T-cell therapies and T-cell therapies in general. It may also be possible to reduce this type of toxicity by targeting antigens that are more restricted in their expression. In B-cell malignancies, for example, we generated CARs specific to κ-light chain of immunoglobulin, since unlike a CD19 CAR-T-cell which will target both normal and malignant B cells equally well, a κ+CAR-T cell will target all the cells of a κ+ malignancy (since the tumor is clonal) while sparing the subset of normal B cells that express λ (54). Nevertheless, to extend CAR-T-cell therapies beyond hematological malignancies in order to treat tumors will likely continue to require targeting of antigens that are inevitably expressed by normal tissues. Preclinical models may be insufficient or inadequate to predict the organ toxicity and in these circumstances alternative safety strategies are required. One approach is to infuse T cells with only transient expression of the CAR, for example after electroporation of mRNA encoding the receptor (198–200). Unlike T cells transduced with a (genome-integrating) vector, in which each daughter cell contains the same transgene, translated to the same level, mRNA transduced T cells express the transgene for a finite period of time (depending on the stability of the mRNA and the translated protein); moreover, levels of expression diminish as the cells divide, and the transcripts become progressively diluted. Since CAR-T cells may expand 1000–10 000-fold over 7–10 days, this dilutional effect may be rapid. The approach is being used at the University of Pennsylvania to test a CAR specific for mesothelin in patients with pancreatic cancer (145).

Controlling SIRS

To reduce the onset or severity of SIRS, investigators are modifying T-cell dose escalation and have introduced the prompt use of antibodies blocking the effects of TNF-α and IL-6.

General reduction in toxicity

While the specific measures outlined above may all be beneficial, the inherent potential of T cells to persist and expand means that the associated toxicities may show corresponding persistence and worsen with time. Thus, there is a strong incentive to use engineered T cells that also express a suicide or safety switch along with the CAR. These cells would then retain their long-term capacity for engraftment, expansion and expression but could be eliminated on demand by the activation of the suicide gene in the event of toxicity. We have selected this approach for our forthcoming clinical trial in which a 3rd CAR targeting the GD2 antigen has been coupled with the inducible caspase9 suicide gene (201, 202). This particular suicide gene can be selectively activated by an otherwise bioinert small molecule (chemical inducer of dimerization). Compared with the more widely used herpes thymidine kinase/ganciclovir approach this system may act more rapidly by induction of apoptosis, and to have reduced immunogenicity since the sequences are all human derived (202, 203).

Although the possibility of rapidly eliminating CAR-T cells in the event of toxicity represents an important consideration for safety, the elimination of these cells also abrogates their anti-tumor effects. This may be problematic if, as seems likely, long term immune surveillance is necessary to prevent tumor relapse. Thus, the ultimate goal is to retain the beneficial anti-tumor effects of CAR-T cells even against antigens that are shared, with normal tissues. One means of accomplishing this is to preferentially express CARs on the T cells only at the tumor site, by exploiting metabolic conditions that are commonly developed within the tumor environment such as hypoxia (204). Targeting multiple antigens on a single cell may also reduce toxicity, by providing the engineered T cells with true pattern-recognition ability. For example, engineering T cells to express two CARs with complementary signaling domains restricts full T-cell activation only to tumor sites at which both antigens are expressed (205–207). These elegant models seem very promising in preclinical experiments, but it remains to be demonstrated whether the benefits can be recapitulated within heterogeneous human malignancies in which the levels and patterns of antigen expression may vary between one cell and another.

Conclusions

CAR-T cells are making the transition from merely 'promising' to being 'effective' treatments for hematological malignancies. As we continue to improve the functionality of the T cells that express chimeric tumor-targeting receptors and enable them to function in the tumor micro-environment, we can anticipate broader application beyond hematological cancers and into solid tumors. With increasing interest in the field from commercial entities we are hopeful that development and clinical implementation of this exciting approach will now accelerate.

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Clinical Cancer Research

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Cancer Therapy: Preclinical

Clinical Cancer Research

T Lymphocytes Redirected against the Chondroitin Sulfate Proteoglycan-4 Control the Growth of Multiple Solid Tumors both *In Vitro* and *In Vivo*

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Abstract

Purpose: Because of its high expression on various types of tumors and its restricted distribution in normal tissues, chondroitin sulfate proteoglycan-4 (CSPG4) represents an attractive target for the antibody-based therapy of several solid tumors. We tested whether T cells transduced with a CSPG4-specific chimeric antigen receptor (CAR) inhibited the growth of CSPG4-expressing tumor cells both *in vitro* and *in vivo*.

Experimental Design: We first independently validated by immunohistochemistry (IHC) the expression of CSPG4 in an extensive panel of tumor arrays and normal tissues as well as queried public gene expression profiling datasets of human tumors. We constructed a second-generation CSPG4-specific CAR also encoding the CD28 costimulatory endodomain (CAR.CSPG4). We then evaluated human T lymphocytes expressing this CAR for their *ex vivo* and *in vivo* antitumor activity against a broad panel of solid tumors.

Results: IHC showed that CSPG4 is highly expressed in melanoma, breast cancer, head and neck squamous cell carcinoma (HNSCC), and mesothelioma. In addition, in silico analysis of microarray expression data identified other important potential tumors expressing this target, including glioblastoma, clear cell renal carcinoma, and sarcomas. T lymphocytes genetically modified with a CSPG4-CAR controlled tumor growth *in vitro* and *in vivo* in NSG mice engrafted with human melanoma, HNSCC, and breast carcinoma cell lines.

Conclusions: CAR.CSPG4-redirected T cells should provide an effective treatment modality for a variety of solid tumors. *Clin Cancer Res; 20(4); 962–71. ©2013 AACR.*

Introduction

Chondroitin sulfate proteoglycan-4 (CSPG4), also known as high-molecular-weight (HMW) melanoma-associated antigen and melanoma-associated chondroitin sulfate proteoglycan (MCSP), is a well-characterized cell surface proteoglycan first identified on human melanoma cells (1). Subsequent studies showed it to be highly expressed on other solid tumors such as mesothelioma (2) and triple negative breast carcinoma (3), all of which often show an aggressive clinical course. In contrast, CSPG4 has a restricted distribution in normal tissues (4).

CSPG4 participates in tumor migration, invasion, angiogenesis, and metastasis (5). It interacts with α4β1 integrins to directly modulate cell adhesion, motility, and metastasis as demonstrated by its ectopic expression in tumor cells (6). Given its restricted expression in normal tissues, high expression on various types of solid tumors and its role in the biology of tumor cells, CSPG4 is an attractive target for immunotherapy.

CSPG4 has been targeted with monoclonal antibodies (mAb) in models of melanoma, mesothelioma, and breast carcinoma, resulting in the inhibition of tumor growth and survival in addition to thwarting the metastatic capability of tumor cells (7). Recent advances in potentiating the antitumor effects of a specific mAb rely on coupling its antigen-binding specificity with the effector function and long-term persistence of T lymphocytes to generate specific chimeric antigen receptors (CAR) (8–10). These molecules are obtained by fusing the extracellular antigen-binding domain of the mAb with the intracellular signaling domains derived from the CD3-ζ chain of the T-cell receptor, in tandem to costimulatory endodomains to support survival and proliferative signals (11–13). Because CAR-modified T cells function independently of a patient's MHC and can

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Translational Relevance

Adoptive transfer of CAR-redirected T lymphocytes represents a promising therapy for patients with lymphoid malignancies. Here we extend the applicability of this strategy to a broad array of solid tumors by targeting the CSPG4 antigen; this antigen is overexpressed by numerous tumor types while having negligible expression in normal tissues. Our study provides preclinical evidence that CSPG4-redirected T cells can control the growth of human melanoma, head and neck squamous cell carcinoma, and breast cancer both *ex vivo* and *in vivo* in xenograft models.

readily be generated for clinical use (14–16), the value of targeting CSPG4 with a CAR-based approach is appealing.

We first validated the expression of CSPG4 in an extensive panel of tumor arrays and normal tissues as well as queried public gene expression profiling datasets of human tumors and confirmed its broad expression. We then generated a CSPG4-specific CAR (CAR.CSPG4) and showed that when expressed by T cells, not only was melanoma effectively targeted *in vitro*, as previously demonstrated (17), but antitumor activity was observed *in vitro* and *in vivo* against many solid tumors including breast carcinoma, head and neck squamous cell carcinoma (HNSCC), and mesothelioma. Redirecting T cells to CSPG4 using CARs may thus represent a robust platform to target multiple solid tumors.

Materials and Methods

Cell lines

The previously described SENMA, CLB, and P1143 tumor cell lines were generated in our laboratory from melanoma biopsies (18). MDA-MB-231 was originally obtained from American Type Culture Collection (ATCC) and authenticated by the analysis of short tandem repeat sequences performed at MD Anderson Cancer Center (Houston, TX). UACC-812, PCI-30, and PHI cell lines were provided by Dr. Ferrone and these cells, when maintained in culture for several passages, retained the same phenotypic expression of CSPG4 as the early cell passages. Previously described melanoma cell lines PLAODE, NE-18732, NE-18588, NE-8959, NE-4405, and NE-371952 were only used to confirm the expression of CSPG4 in a broad array of melanoma cell lines (18). All these cells, including SENMA, CLB, and P1143, when maintained in culture for several passages, retained the same phenotypic expression of CSPG4 as the early cell passages. SENMA, CLB, UACC-812, MDA-MB-231, and PCI-30 cell lines were cultured in Dulbecco's Modified Eagle Medium (Invitrogen) or RPMI 1640 (P1143, UACC-812, and PHI; Cambrex) medium supplemented with 10% heat inactivated fetal calf serum (FCS; HyClone, Thermo Fisher Scientific Inc.), 200 IU/mL penicillin, 200 mg/mL streptomycin (Invitrogen), and 2 mmol/L GlutaMAX (Invitrogen) at 37°C in a 5% CO_2 atmosphere. Tumor cell lines were transduced with a γ retroviral vector-encoding eGFP to obtain GFP^+ tumor cells (>98% GFP^+). Primary epithelial cells from normal small airway, kidney, and prostate were purchased from ATCC and kept in culture according to ATCC recommendation.

Tissue microarrays and immunohistochemistry

Antigen retrieval was performed by placing the samples in 1× Dako Citrate Buffer followed by incubation at 90°C in a pressure cooker for 45 minutes. After blocking with normal goat serum diluted in Tris-buffered saline, samples were incubated with the CSPG4 mAb [Abcam; anti-NG2 antibody (LHM 2), ref. no. ab104535; 1:300 dilution] either overnight at 4°C or at room temperature for 1 hour. Detection of CSPG4 was then assessed using the VECTASTAIN ABC Kit (Vector Laboratories, Inc., ref. no. PK-4001) following the manufacturer's protocol. Tissue arrays were obtained from Cybrdi Inc. for breast cancer (CC08-10-001) and HNSCC (CC34-01-001), whereas melanoma (ME2082b), neuroblastoma (MC602), mesothelioma (T392), and normal tissue (FDA 808b) arrays were obtained from US Biomax, Inc. Each array contained a range of 4 to 192 cores of tumor or 5 to 18 cores of normal tissue samples in duplicate, triplicate, or quadruplicate in the case of mesothelioma. Expression of CSPG4 in tumor cells was scored in blind fashion by the pathologist Dr. M. Ittmann based on both intensity (0–3+) and extent of staining (1–3+). A multiplicative staining score was calculated by multiplying the intensity and extent scores to yield scores on a 10-point scale from 0 to 9 (19). In microarrays with multiple cores per patient, the individual scores were averaged to obtain a final score. In some cores, tumor was not identified because of artifacts. In the vast majority of cases, immunohistochemistry (IHC) showed uniform staining (3+) within a given core and in most cases cores from different patients were highly concordant. Areas of necrosis or acellular keratin were not included in the scoring. Cases were divided based on staining scores into 3 groups: negative/weak (0–3), moderate (4–6), or strong (7–9).

Generation of the CSPG4-specific CAR and transduction of T lymphocytes

The hybridoma 763.74 was generated from a BALB/c mouse immunized with cultured human melanoma cells (20). The scFv 763.74 was isolated from the hybridoma (21) and then cloned in frame with the human IgG1-CH_2CH_3 domains, the CD28 costimulatory endodomain, and the CD3ζ chain into the SFG retroviral backbone (CAR.CSPG4), as previously described (22, 23). The control CAR specific for the CD19 antigen (CAR.CD19) has been previously described (13). Transient retroviral supernatant was generated by cotransfection of 293T cells with the RD114 envelope (RDF plasmid), the MoMLV gag-pol (PegPam3-e plasmid), and the retroviral vector, as previously described (23). For the generation of CAR-T cells, peripheral blood mononuclear cells (PBMC) were isolated from buffy coat preparations (Gulf Coast Regional Blood Center, Houston, TX) using Ficoll-Paque (Amersham Biosciences). PBMCs were activated with OKT3 and CD28 (BD Biosciences

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PharMingen) mAbs, transduced with the retroviral supernatant by day 3 of culture and then expanded in complete medium containing 45% RPMI 1640 and 45% Click's medium (Irvine Scientific) supplemented with 10% FCS, 100 IU/mL penicillin, 100 mg/mL streptomycin, and 2 mmol/L GlutaMAX. Cells were fed with interleukin (IL)-2 (50 U/mL; PeproTech) twice a week for 2 weeks (23).

Flow cytometry

Conjugated CD3, CD4, CD8, CD45RO, CD62L, and CCR7 mAbs (BD Biosciences) were used to identify T lymphocytes, whereas the CSPG4 mAb (Miltenyi-Biotech Inc.) was used to label tumor cells. CAR expression in T lymphocytes was assessed using an antibody recognizing the human IgG1-CH_2CH_3 fragment (Jackson ImmunoResearch) (23). Analyses were performed on a FACsCaliber flow cytometer using the BDFACs CellQuestPro software (BD Biosciences).

Cytotoxicity and coculture assays

The cytotoxic activity of control and CAR.CSPG4$^+$ T lymphocytes was determined using a standard ^{51}Cr release assay at different effector-to-target (E:T; 40:1, 20:1, 10:1, and 5:1) ratios using a γ counter (Perkin-Elmer) (23). For the coculture experiments, control and CAR.CSPG4$^+$ T lymphocytes were plated at 1×10^6 cells/well in 24-well plates at different E:T ratios according to the kinetic growth of each tumor cell line. Tumor cell lines with a slow kinetic growth were plated at higher tumor ratio (T cells:tumor cells 3:1) compared with tumor cell lines with a fast kinetic growth (T cells:tumor cells 5:1). Supernatant was collected at 24 hours of culture to measure IFN-γ and IL-2 release using specific ELISAs (R&D Systems). Following 72 hours of culture at 37°C, adherent tumor cells and T cells were collected and residual tumor cells and T cells assessed by fluorescence-activated cell-sorting (FACS) analysis based on GFP and CD3 expression, respectively.

Carboxyfluorescein diacetate succinimidyl ester assay

One week posttransduction, control and CAR.CSPG4$^+$ T lymphocytes were labeled with 1.5 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) and plated with irradiated tumor target (SENMA) at an E:T ratio of 5:1. CFSE dilution was measured on CD4$^+$ and CD8$^+$ cells by flow cytometry by day 4 of coculture.

Xenogenic mouse models

In vivo experiments were performed in accordance with Baylor College of Medicine's Animal Husbandry guidelines. Antitumor activity of control and CAR.CSPG4$^+$ T lymphocytes was evaluated using NOG/SCID/$\gamma c^{-/-}$ (NSG) mice (Jackson Lab, Bar Harbor, ME) engrafted with tumor cells. Eight- to nine-week-old mice were subcutaneously injected with 0.5×10^6 SENMA, 3×10^6 UACC-812, or 3×10^6 PCI-30 cells resuspended in Matrigel (BD Biosciences). On days 4, 6, and 8 following tumor cell injection, 1×10^7 control or CAR.CSPG4$^+$ T lymphocytes were injected intravenously by tail vein. In summary, for the melanoma xenograft model 3 different preparations of CAR.CSPG4 T cells were generated from 3 different donors. Three doses, given 2 days apart, of 1×10^7 were infused intravenously into 5 mice per group. In total, 15 animals were treated for each group. The endpoint of the experiment was to examine differences in tumor volume up to day 30 after tumor injection. For the xenograft models of breast cancer and HNSCC, 2 different preparations of T cells generated from 2 different donors were used. Two doses, given 2 days apart, of 1×10^7 were infused intravenously into 5 mice per group. In total, 10 animals were treated per group. In all tumor models, mice were sacrificed at 30 days or in accordance with our institution's guidelines for the handling of sick animals. Weekly manual caliper measurements were performed posttreatment to evaluate tumor growth. Tumor volume was calculated using the modified ellipsoidal formula: tumor volume (mm^3) = $(\text{width})^2 \times \text{length}/2$.

Statistical analysis

In vitro data are presented as mean ± SD and a paired Student *t* test was used to determine statistical significance. The *in vivo* data are presented as mean ± SEM and a paired Student *t* test was used to identify significant differences between CAR- and control-treated groups. Public gene expression profiling datasets of human tumors were queried for *CSPG4*, including data from The Cancer Genome Atlas (TCGA Data Portal; http://tcga-data.nci.nih.gov/tcga), Bittner multicancer dataset (unpublished, from http://www.oncomine.org/www.oncomine.org; refs. 26, 27) and GeneAtlas U133A dataset (http://biogps.org).

Results

CSPG4 is expressed on a variety of solid tumors

As CSPG4 was originally identified as a melanoma-associated antigen, we first independently validated its expression using IHC in a melanoma tissue array containing multiple primary cutaneous and visceral melanomas and metastatic lesions. Examples of either strong or negative/low staining are shown in Fig. 1A. Consistent expression of the antigen was documented in all types of lesions, regardless of their primary or metastatic origin, or their cutaneous and visceral source. We therefore analyzed melanomas as a whole group. Overall, 59% of melanomas showed strong staining and 25% displayed moderate staining (Fig. 1B). We then extended the analysis to include multiple samples of additional solid tumors including breast cancer, HNSCC, neuroblastoma, and mesothelioma. For the breast cancer array, staining was seen in invasive ductal and lobular carcinomas as well as in the small number of Paget disease and ductal *in situ* carcinoma cases present on the array. Staining for representative invasive ductal carcinomas (either strong or negative/low) are shown in Fig. 1A and summarized in Fig. 1B, whereas other lesions were not sufficient in number for a comparative analysis. Staining for CSPG4 in invasive ductal carcinoma was remarkable with 77% of cases showing moderate or strong staining. HNSCC most predominantly (50%) expressed moderate staining for CSPG4, with only 20% showing strong staining. Although neuroblastoma exhibited the weakest overall

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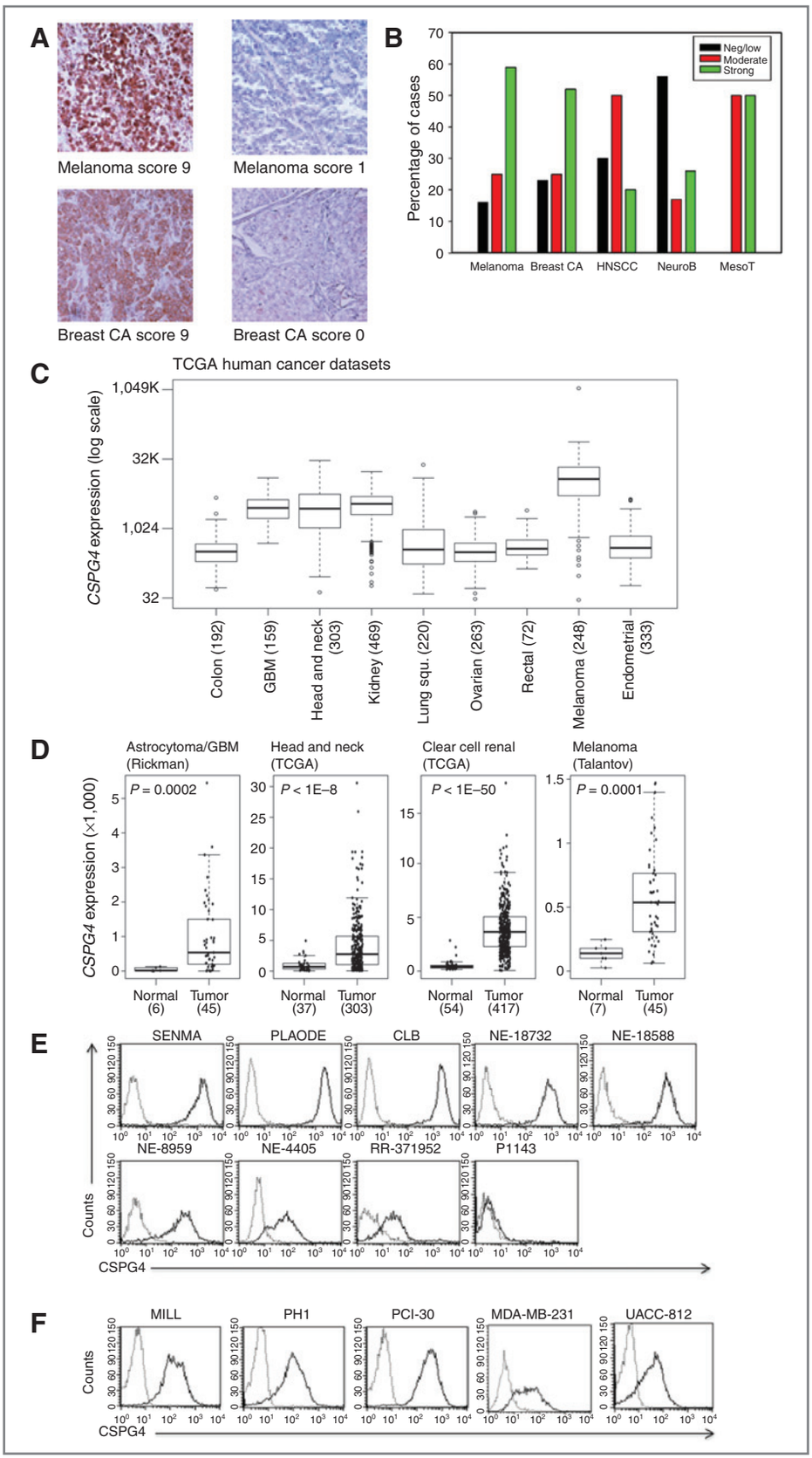

Figure 1. CSPG4 expression in primary solid tumors and tumor-derived cell lines. A, representative IHC and scoring of analyzed solid tumor tissue arrays. Representative melanoma and breast carcinoma samples are shown at ×200 magnification. B, scoring summary of a panel of solid tumors that includes melanoma, breast carcinoma (Breast CA), HNSCC, neuroblastoma (NeuroB), and mesothelioma (MesoT). C, *CSPG4* mRNA expression by TCGA in a variety of solid tumors. Box plots show median, 25%–75% range, 5%–95% range, and minimum/maximum. D, *CSPG4* mRNA expression analysis, comparing tumor versus corresponding normal tissues, for astrocytoma/glioblastoma (GBM), HNSCC, clear cell renal carcinoma, and melanoma. Indicated *P* values were calculated by *t* test. E, CSPG4 expression in the indicated array of melanoma cell lines as assessed by flow cytometry (FACS). F, FACS analysis of CSPG4 expression in the selected mesothelioma (MILL and PHI), HNSCC (PCI-30), and breast cancer–derived (MDA-MB-231 and UACC-812) cells lines. Dotted and bold lines indicate isotype and CSPG4 mAbs, respectively.

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staining, there was still a fraction of cases with moderate to strong expression. Finally, despite the limited number of mesotheliomas, these lesions all consistently expressed CSPG4. We concluded that, at the protein level, all these malignancies exhibited variable but in most cases significant expression of CSPG4. To examine the expression of CSPG4 in a broader array of tumors, we examined publically available databases for mRNA expression data. As shown in Fig. 1C, examination of TCGA datasets showed overexpression of CSPG4 transcripts in melanoma and in glioma as anticipated based on the previously reported expression of the protein (28). Concordant with our protein expression data, CSPG4 mRNA expression was increased in HNSCC. We also found increased mRNA expression in clear cell renal carcinomas by *in silico* analysis, and overall, despite some intratumor variability, significant increased mRNA levels in all these tumor types relative to the corresponding normal tissues (Fig. 1D). Examination of the large Bittner multicancer dataset (www.oncomine.org) confirmed high CSPG4 mRNA expression in melanoma, clear cell renal carcinoma, HNSCC, multiple sarcoma types (chondrosarcoma, leiomyosarcoma, liposarcoma), gastrointestinal stromal tumors, skin, and vulvar squamous cell carcinomas (Supplementary Fig. S1). Of note, several sarcoma cell lines have been previously reported to express CSPG4 protein (3). A number of other common malignancies such as colorectal, ovarian, and endometrial carcinoma did not show increased CSPG4 transcripts, consistent with the mRNA expression from the TCGA datasets. CSPG4 protein expression in an array of normal tissues was negative (Supplementary Fig. S2). In addition to the normal tissues represented in Supplementary Fig. S2, we evaluated CSPG4 expression on a total of 33 different types of tissues, all of which were negative. Using the public Novartis GeneAtlas (http://biogps.org) and TCGA databases, CSPG4 mRNA expression was observed in a number of normal tissues. However, the levels of expression are remarkably lower than those of cancer tissues (Supplementary Fig. S3).

We next examined CSPG4 expression in a series of cell lines from a variety of tumor types analyzed above. Expression of CSPG4 was detected in 8 of the 9 melanoma cell lines screened (Fig. 1E). Importantly, CSPG4 was detected on tumor cell lines representative of the above-identified solid tumors such as mesothelioma (MILL and PHI), HNSCC (PCI-30), and breast cancer [MDA-MB-231 (adenocarcinoma) and UACC-812 (ductal carcinoma); Fig. 1F], all consistent with our analysis of human tumor samples.

T lymphocytes expressing the CSPG4-specific CAR are cytotoxic against CSPG4$^+$ tumor cell lines but not against primary normal tissues

To target CSPG4$^+$ tumors we generated a CSPG4-specific CAR containing the CD28 costimulatory endodomain (CAR.CSPG4; Fig. 2A). T lymphocytes from 4 healthy donors were engineered to express the CAR.CSPG4 using a γ retroviral vector. Transduction efficiency was 80% ± 3%, and both CD4 and CD8 T cells stably expressed the CAR (26% ± 9% and 51% ± 16%, respectively), as assessed by phenotypic analysis by day 7 of culture (Fig. 2B). The majority of CAR.CSPG4$^+$ T cells were CD45RO$^+$ (76% ± 7%) and a fraction retained CD62L expression (51% ± 7%) and CCR7 (13% ± 2%), indicating that they were mainly composed of effector-memory T cells (Fig. 2C). The expression of CAR.CSPG4 by T cells was comparable to that obtained with a previously described CD19-specific CAR (CAR.CD19) (Supplementary Fig. S4; ref. 29), which was used as an irrelevant-CAR control population.

Cytotoxic activity of control and CAR.CSPG4$^+$ T cells, after 1 to 2 weeks of culture, was assessed against K562, to measure natural killer cell-mediated activity, and against the melanoma-derived cells lines P1143 (as CSPG4$^-$ target) and SENMA (as CSPG4$^+$ target; Fig. 1E) at various E:T ratios (Fig. 3A). CAR.CSPG4$^+$ but not control T lymphocytes significantly lysed the CSPG4$^+$ target (59% ± 5% vs. 11% ± 8% at 20:1 ratio; $P < 0.01$), whereas both CAR.CSPG4$^+$ and control T cells showed minimal activity against K562 (12% ± 9% vs. 13% ± 11%) and the CSPG4$^-$ target (<10% in both cases). The antitumor activity of CAR.CSPG4$^+$ T lymphocytes was also evaluated in a 72-hour coculture assay (Fig. 3B and C). CAR.CSPG4$^+$ and control T lymphocytes were cocultured with GFP-expressing tumor cell lines at an E:T ratio ranging from 5:1 to 3:1 according to the kinetic growth of each cell line. CAR.CSPG4$^+$ T cells significantly controlled the growth of all CSPG4$^+$ cell lines tested: SENMA (residual tumor cells = 0.1% ± 0.06%), CLB (0.1% ± 0.1%), UACC-812 (6% ± 6%), MILL (3% ± 5%), MDA-MB-231 (3% ± 3%), PHI (4% ± 3%), and PCI-30 (0.5% ± 0.5%), but not of the CSPG4$^-$ target P1143 (residual tumor cells 38% ± 10%). As expected, all tumor cell lines tested rapidly grow in the presence of control T lymphocytes (residual tumor cells for: SENMA = 62% ± 3%, CLB = 70% ± 6%, UACC-812 = 47% ± 15%, MILL = 50% ± 8%, MDA-MB-231 = 42% ± 11%, PHI = 29% ± 6%, PCI-30 = 17% ± 3%, and P1143 = 45% ± 10%). In all cases, the effects of CAR.CSPG4$^+$ T cells were significantly greater than those of control T cells (from $P < 0.05$ to $P < 0.001$). T cells expressing the control CAR.CD19 showed cytotoxic activity neither against CSPG4$^+$ nor CSPG4 targets (Supplementary Fig. S4). As illustrated in Fig. 3, commercially available primary normal epithelial cell lines (small airway, kidney, and prostate) derived from tissuses found to express low levels of CSPG4 mRNA (Supplementary Fig. S3) did not express detectable levels of the protein by flow cytometry (Fig. 3D), and were not lysed by CAR.CSPG4$^+$ T cells when tested in ^{51}Cr release assays (Fig. 3E).

CAR.CSPG4$^+$ T lymphocytes secrete Th1 cytokines and proliferate in response to CSPG4$^+$ tumors

Because CAR.CSPG4 contains the CD28 costimulatory endodomain, we studied CAR.CSPG4$^+$ T lymphocyte proliferation in response to CSPG4$^+$ tumor cells using a CFSE dilution assay. When CFSE-labeled control and CAR.CSPG4$^+$ T cells were cultured with irradiated SENMA tumor cells for 96 hours, a significant CFSE dilution occurred for CAR.CSPG4$^+$ T cells, with both CD4 and CD8 T cells proliferating at a higher percentage (66% ± 12% and

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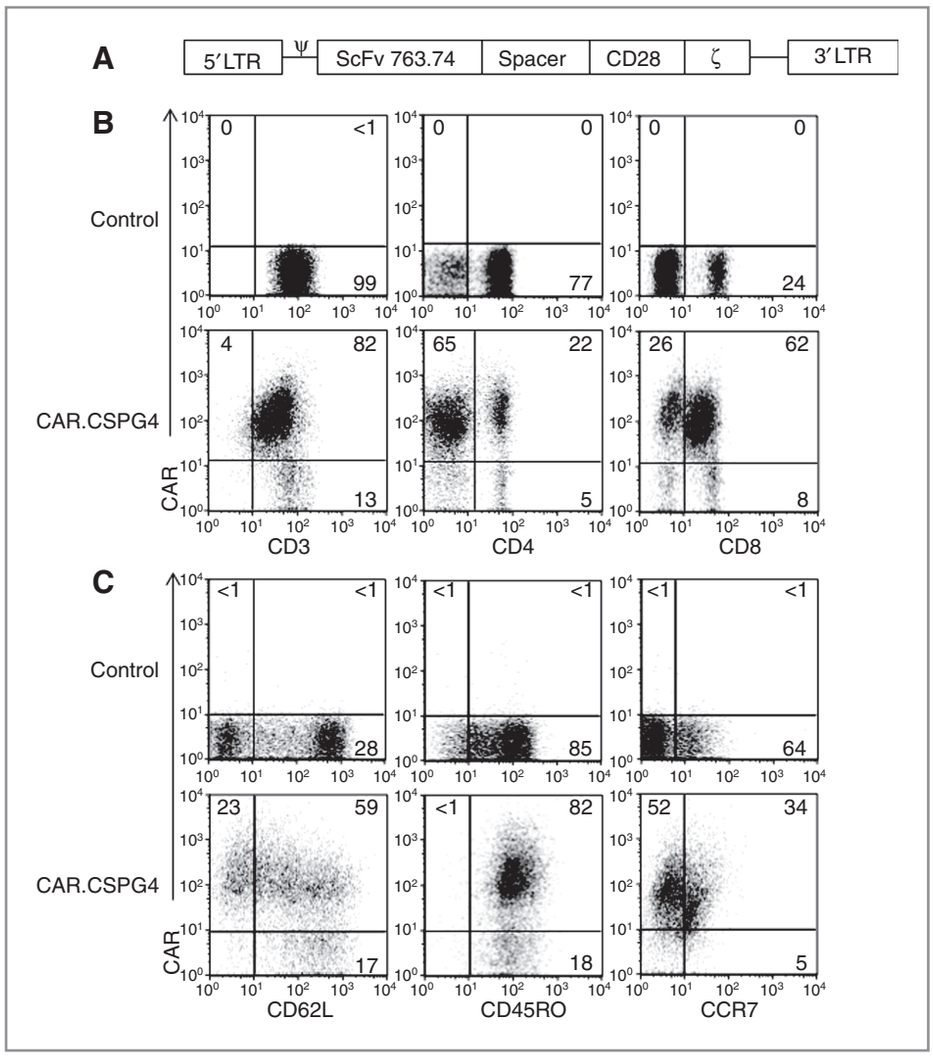

Figure 2. Expression and function of CAR.CSPG4 in T cells. A, schematic representation of the retroviral vector encoding the CSPG4-specific CAR. The CAR incorporates the CD28 costimulatory endodomain. B, representative FACS analysis showing the expression of the CAR in CD3, CD4, and CD8 T cells after retroviral transduction. C, representative expression of the CD62L, CD45RO, and CCR7 markers on control and CAR.CSPG4$^+$ T cells by flow cytometry on day 14 of culture. Numbers represent percentages of cells per quadrant.

68% ± 8%, respectively) compared with control CD4 and CD8 T cells (8% ± 7% and 14% ± 10%, respectively; $P < 0.05$ and $P < 0.01$, respectively; Fig. 4A and B). T cells transduced with the control CAR.CD19 also containing the CD28 endodomain did not show significant proliferation in response to CSPG4$^+$ targets (Supplementary Fig. S4). We also evaluated whether the inclusion of a "late" costimulatory endodomain, such as 4-1BB, in addition to CD28 (third-generation construct) provided these T cells with additional proliferative and cytotoxic activity, but found no further benefits (Supplementary Fig. S5).

We finally quantified the IL-2 and IFN-γ cytokines released in response to the antigen, by coculturing control and CAR.CSPG4$^+$ T lymphocytes with CSPG4$^+$ or CSPG4$^-$ tumor cells. As expected CAR.CSPG4$^+$ T lymphocytes secreted significantly more IL-2 than control T cells only in the presence of CSPG4$^+$ tumor cells (Fig. 4C). A positive trend, although not statistically significant, was observed when CAR.CSPG4$^+$ T lymphocytes were cultured with the CSPG4$^+$ tumor cell lines PHI, MILL, UACC-812, and MDA-MB-231 (data not shown). Similarly IFN-γ production was significantly higher than control T cells when CAR.CSPG4$^+$ T lymphocytes were cultured with CSPG4$^+$ tumor cells (Fig. 4D).

CAR.CSPG4$^+$ T lymphocytes control the growth of human melanoma, HNSCC, and breast carcinoma cells engrafted in immunodeficient mice

To assess the *in vivo* relevance of our *in vitro* results, we engrafted NSG mice subcutaneously with cell lines derived from representative melanoma tumor (SENMA), HNSCC (PCI-30), or breast carcinoma (UACC-812). Four to seven days later (depending on the kinetics of the tumor growth), mice were infused via tail vein injection with either control or CAR.CSPG4$^+$ T lymphocytes, and tumor growth quantified by sequential tumor volume measurements. In all 3 models, CAR.CSPG4$^+$ T lymphocytes inhibited tumor growth significantly better than control T lymphocytes (Fig. 5). By day 30, melanoma tumors reached a volume of 879 ± 124 mm^3 in mice receiving CAR.CSPG4$^+$ T lymphocytes versus 8,359 ± 958 mm^3 in mice receiving

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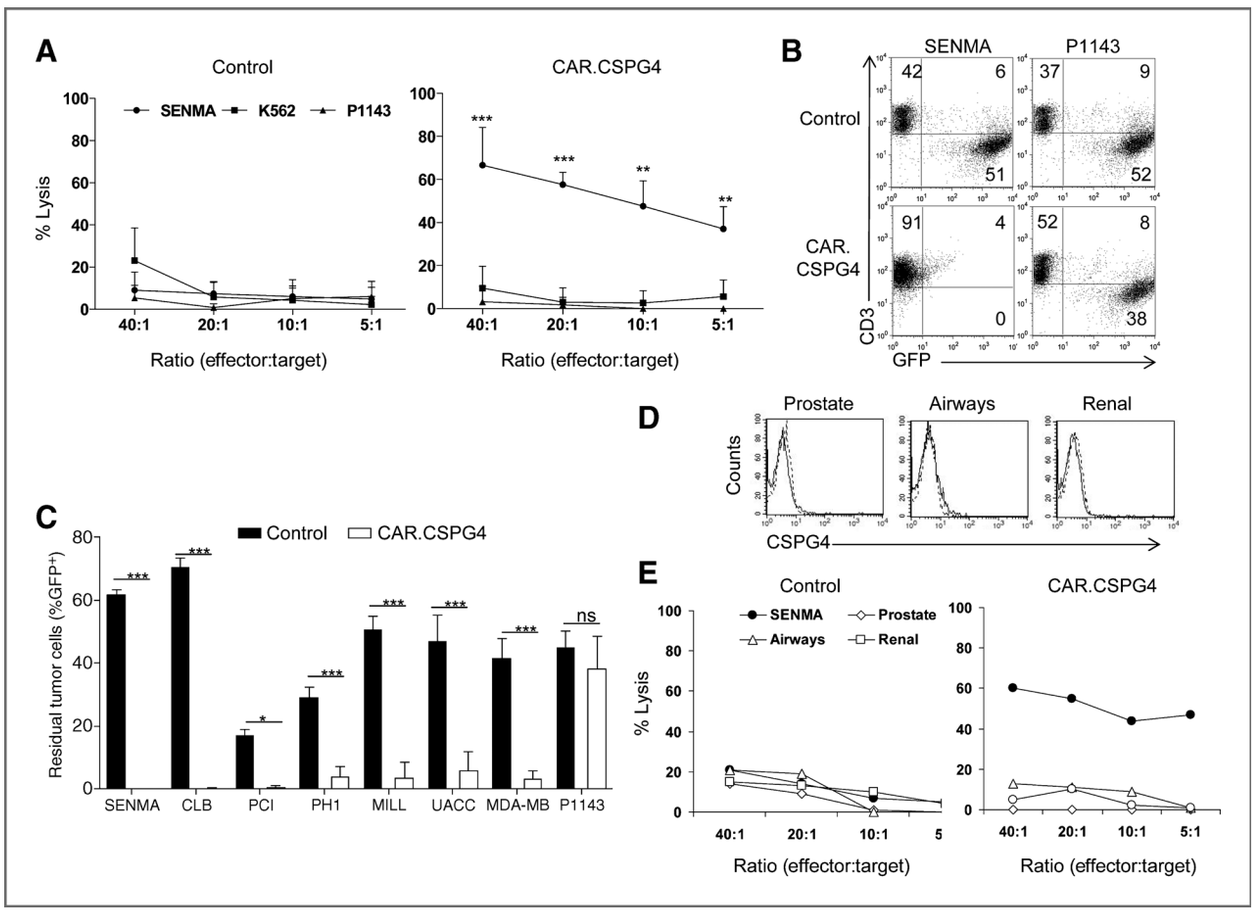

Figure 3. Cytotoxic function of CAR.CSPG4$^+$ T cells against CSPG4$^+$ tumors but not against epithelial cells from lung, kidney, and prostate. A, cytotoxic activity of control T cells and CAR.CSPG4$^+$ T cells evaluated in a 6-hour ^{51}Cr release assay. Target cells used were the CSPG4$^+$ tumor cell line (SENMA), CSPG4$^-$ target cell line (P1143), and K562 to quantify natural killer activity. Data, averages and SD results of T cells from 4 donors. B, coculture experiments of control and CAR.CSPG4$^+$ T cells with GFP$^+$ tumor cell lines, assessed by flow cytometry 72 hours later. The plots describe a representative experiment of T cells cocultured with SENMA (CSPG4$^+$ target) or P1143 (CSPG4$^-$ target). Numbers represent percentages of cells per quadrant. C, summary of coculture experiments of control and CAR.CSPG4$^+$ T cells against a panel of CSPG4$^+$ tumor targets. Data, averages ± SD of 4 donors. *, $P < 0.05$; ***, $P < 0.001$. D, FACS analysis of CSPG4 expression in primary epithelial cells derived from normal small airway, kidney, and prostate. Dotted and bold lines indicate isotype and CSPG4 mAbs, respectively. E, cytotoxic activity of control T cells and CAR.CSPG4$^+$ T cells from a representative donor of 2 independent experiments evaluated in a 5-hour ^{51}Cr release assay against these normal epithelial cells.

control T cells ($P < 0.001$; Fig. 5A), and this corresponded to improved overall survival (Supplementary Fig. S6). Although HNSCC and breast carcinoma tumors were not as aggressive as melanoma *in vivo*, we observed that in both models CAR.CSPG4$^+$ T lymphocytes controlled tumor growth. By day 30, the size of HNSCC tumors was 19 ± 10 mm^3 in treated mice versus 190 ± 75 mm^3 in control mice ($P < 0.001$; Fig. 5B) and the size of breast carcinoma tumors was 28 ± 13 mm^3 in treated mice versus 166 ± 64 mm^3 in control mice ($P < 0.001$; Fig. 5C).

Discussion

The involvement in several signaling pathways associated with cell proliferation, survival, migration, and suggested high expression in various types of cancers highlight the critical role that CSPG4 has in promoting tumor growth and simultaneously make it an attractive target for immunotherapy. By IHC, we independently validated CSPG4 protein expression in several solid tumors with poor prognosis, such as melanoma, breast cancer, mesothelioma, and HNSCC. *In silico* analysis of microarray expression data confirmed overexpression of CSPG4 in tumors that we validated by IHC as compared with normal tissues, and also disclosed CSPG4 overexpression in other important malignancies including glioblastoma, clear cell renal carcinoma, and sarcomas suggesting that targeting this antigen may have a major impact on a broad array of solid tumors.

Because CSPG4-specific mAbs can control tumor growth of CSPG4$^+$ tumor cells, in both melanoma and breast cancer tumor models (7), we proposed to improve the therapeutic benefits of this antibody-based approach by generating a CAR that targets the CSPG4 molecule. In contrast to mAb-based therapy, CAR-T cells should produce

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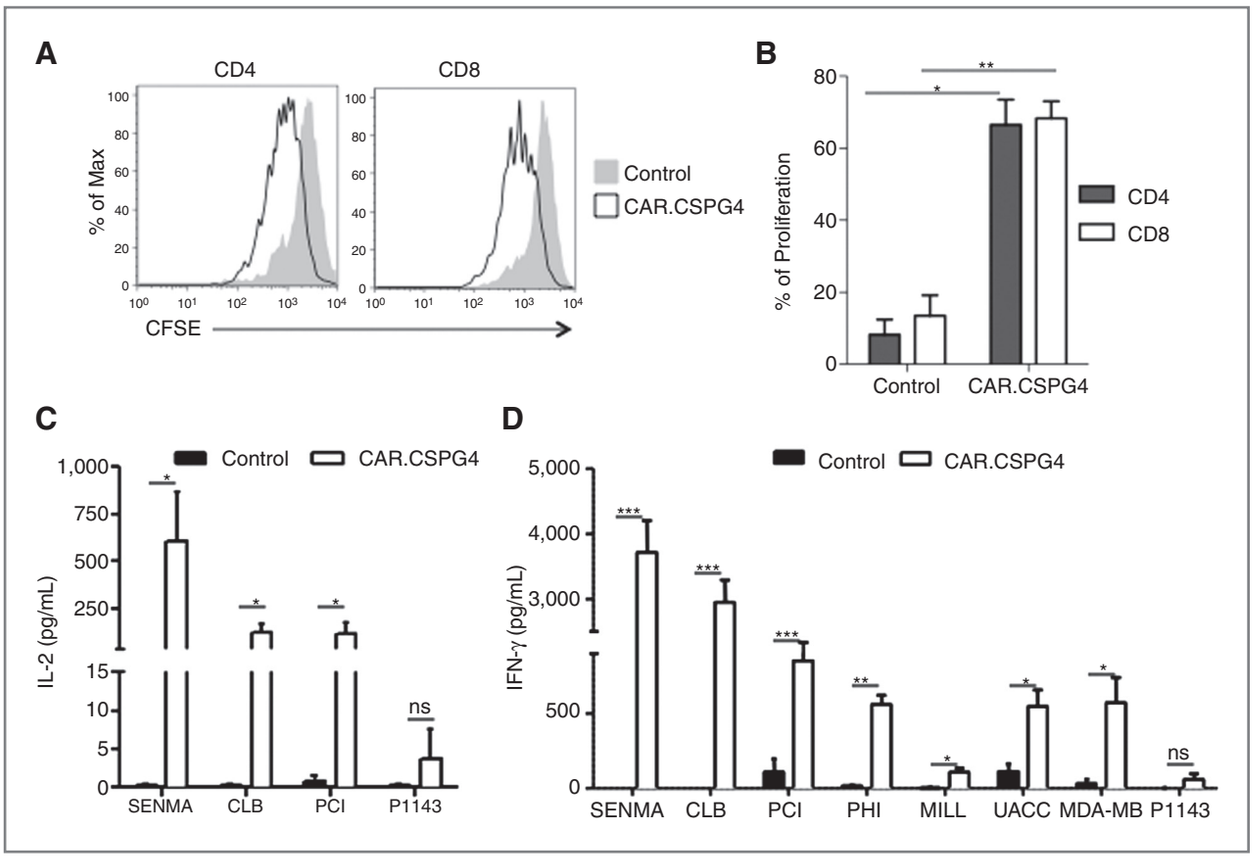

Figure 4. T lymphocytes transduced with CAR.CSPG4 proliferate and release IL-2 and IFN-γ upon specific antigen engagement. A, control and CAR.CSPG4$^+$ T cells, labeled with CFSE, were stimulated with irradiated CSPG4$^+$ (SENMA) tumor target. The panel illustrates the CFSE dilution in CD4- or CD8-gated T cells after 96 hours of culture for a representative donor. B, summary of 3 independent CFSE dilution assays. Data, mean ± SD. C, IL-2 cytokine-release assessment using specific ELISA by T lymphocytes transduced with CAR.CSPG4 and control T cells 24 hours after coculture (E:T ratio 5:1) with either CSPG4$^+$ tumors or CSPG4$^-$ target cells (P1143). Results of 5 experiments are presented with mean ± SD. D, illustrates the detection of IFN-γ in the same culture supernatant. Results of 5 experiments with mean ± SD are shown. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

long-lasting effects, as engineered T cells can expand at the tumor site upon antigen stimulation if an appropriate costimulatory endodomain, derived from CD28, CD137, or CD134, is incorporated within the CAR (13, 30). In contrast to a previous report (21), we found that the CSPG4-specific CAR obtained from the same 763.74 single chain has potent antitumor activity. We traced these striking differences to 2 critical components we have introduced in our construct. First, the scFv in our CAR is coupled with the CD3-ζ endodomain of the TCR rather than the FcεRI-γ chain, which is known to promote a much weaker and less durable signaling (31, 32). Second, we incorporated the CD28 costimulatory endodomain within the CAR, to accomplish sustained IL-2 production and proliferation in response to CSPG4$^+$ tumor cells, thus recapitulating previous observations for other CAR molecules (13). Of note, the inclusion of a second costimulatory endodomain derived from CD137 did not further improve the function of our CAR *in vitro*, supporting the concept that there is no single optimal configuration that is applicable to all CAR molecules, but that CAR receptor optimization remains largely empirical and required for each molecule.

The most critical improvement in the field by our work is the applicability of CAR.CSPG4$^+$ T cells not only to target melanoma (17), but more broadly to other solid tumors generally characterized by poor prognosis with conventional treatments such as breast carcinoma, HNSCC, and mesothelioma. We demonstrated that CAR.CSPG4$^+$ T cells produce IFN-γ and promote tumor elimination not only when challenged with tumor cells with high CSPG4 expression but also with tumor cell lines characterized by moderate/low CSPG4 expression, such as the breast carcinoma tumor cell lines UACC-812 and MBA-MB-231. This further supports the advantages of antibody specificity coupled with the T-cell effector function, as mastered by CAR-modified T cells, which can target tumors neglected by naked corresponding antibodies because of the suboptimal expression of the targeted antigen (33, 34).

Antitumor effects mediated by CAR.CSPG4$^+$ T cells significantly limit tumor growth in xenograft mouse models of melanoma, HNSCC, and breast carcinoma, strongly validating our *in vitro* findings. The lack of sustained and complete tumor eradication in these models was not caused by selection of CSPG4-negative tumor cells, as harvested

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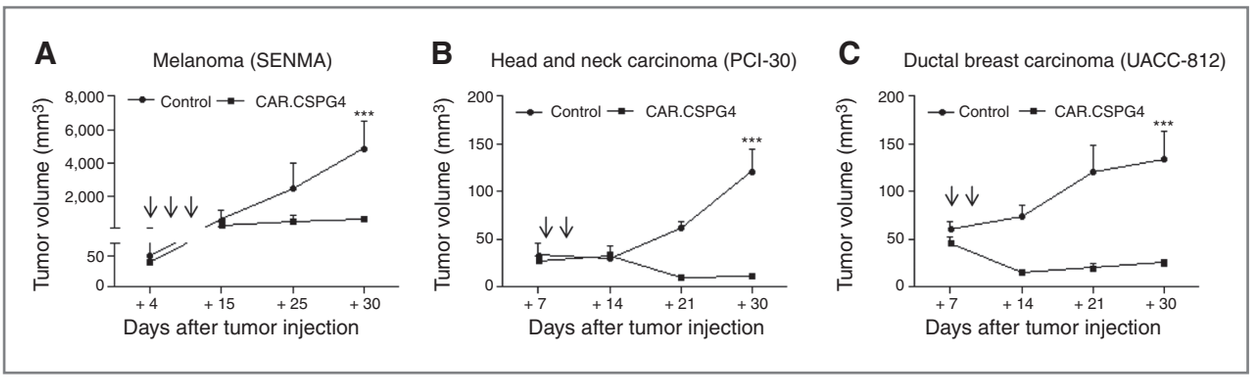

Figure 5. CAR.CSPG4$^+$ T lymphocytes control tumor growth *in vivo*. A–C, tumor growth, assessed by caliper measurement, of NSG mice engrafted subcutaneously with melanoma (SENMA; A), HNSCC (PCI-30; B), or breast carcinoma (UACC-812; C) cell lines and infused intravenously with either CAR.CSPG4$^+$ (closed squares) or control (closed circles) T lymphocytes. Arrows indicate T-cell infusions. Shown are mean ± SD from 15 mice per group (3 independent experiments) for the melanoma model and 10 mice per group (2 independent experiments) for the HNSCC model and breast carcinoma models. ***, $P < 0.001$.

tumors retained the expression of the antigen (Supplementary Fig. S6), but conversely are likely to be attributed to an intrinsic limitation of the models, as T cells do not persist long term in these immunodeficient mice (Supplementary Fig. S6).

To fully translate this approach, the differential expression of CSPG4 in tumor cells versus normal tissues needs to be ensured to limit potential toxicities (35, 36). We found the expression of CSPG4 absent or negligible in normal tissue arrays as assessed by IHC. The analysis of publically available datasets indicates that there is some level of CSPG4 mRNA expression in several normal tissues. However, when we compared normal tissues with cancer tissues, the cancers show consistent and dramatically higher expression of CSPG4 at mRNA levels. Our *in vitro* analyses illustrate that primary epithelial cells derived from some of these tissues do not express significant amount of the protein and are not targeted by CAR.CSPG4$^+$ T cells. Even though the tissue screening, bioinformatics analysis, and lack of toxicity by *in vitro* experiments support the relevance of CSGP4 as a targetable antigen in cancer patients, we cannot fully exclude that the low levels of mRNA in normal tissues, as reported in public datasets, may promote sufficient protein expression in specific physiologic conditions to become a target for CSPG4-specific CAR-T cells. The clinical translation of this approach may thus benefit from the inclusion of a suicide gene within the vector cassette, to allow the rapid elimination of CAR-modified T cells in case of undesired toxicity (29, 37).

In summary, we provide ample data to support the use of CAR.CSPG4$^+$ T cells to treat a broad range of solid CSPG4$^+$ tumors for which the prognosis remains poor with conventional treatments. The combination of this approach with other biologic agents may further increase their activity and thus clinical benefits.

Disclosure of Potential Conflicts of Interest

Center for Cell and Gene Therapy has a collaborative research agreement with Celgene and bluebird bio.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Caruana, M. Del Vecchio, C. Geldres, B. Savoldo, V. Hoyos, E. Yvon, M.M. Ittmann, G. Dotti
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Geldres, B. Savoldo, V. Hoyos, C.J. Creighton, M.M. Ittmann, G. Dotti
Writing, review, and/or revision of the manuscript: C. Geldres, B. Savoldo, M. Del Vecchio, C.J. Creighton, M.M. Ittmann, S. Ferrone, G. Dotti
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Dotti
Study supervision: G. Dotti

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Chapter 17

Genetic Modification of Cytotoxic T Lymphocytes to Express Cytokine Receptors

Serena K. Perna, Barbara Savoldo, and Gianpietro Dotti

Abstract

Adoptive transfer of tumor-infiltrating lymphocytes (TIL) or antigen-specific cytotoxic T lymphocytes (CTL) is safe and can be effective in cancer patients. Achievement of clinical responses in these patients is associated with the in vivo expansion and persistence of the transferred T lymphocytes. For this reason, recombinant human interleukin-2 (IL-2) is frequently used to support the in vivo survival of T lymphocytes infused into patients. However, IL-2 also causes important side effects. Thus, alternative strategies are highly demanded to limit cytokine-related off-target effects and to redirect the responsiveness of specific T-cell subsets to selected cytokines. Interleukin-7 (IL-7) is a promising alternative cytokine as it possesses the above mentioned properties. However, because its receptor is downregulated in ex vivo-expanded T cells, methods are required to restore their responsiveness to this homeostatic cytokine. In this chapter, we describe the methodology to obtain the ectopic expression of IL-7 receptor alpha (IL-7Rα) in antigen-specific CTL, using Epstein–Barr virus-specific CTL (EBV-CTL), as a model.

Key words Immunotherapy, Cytotoxic T lymphocytes, Cytokine receptors, Interleukin-2, Interleukin-7, Adoptive transfer of T lymphocytes

1 Introduction

Adoptive transfer of tumor-infiltrating lymphocytes (TIL) or antigen-specific cytotoxic T lymphocytes (CTL) expanded ex vivo has proven safe and effective for the treatment of melanoma and virus-associated malignancies, such as Epstein–Barr virus-mediated posttransplant lymphomas (PTLD), and EBV-associated Hodgkin's lymphomas and nasopharyngeal carcinomas [1–4].

A prerequisite for the adoptive transfer of TIL or antigen-specific CTL is the ex vivo expansion of specific T-cell precursors from tumor biopsies (in case of TIL) or from peripheral blood (in case of antigen-specific CTL) of cancer patients. Despite the variety of protocols available for the ex vivo expansion of TIL and antigen-specific CTL, a common element is the use of gamma-chain (γc) cytokines such as IL-2, IL-7, and interleukin-15 (IL-15)

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in the culture media to obtain robust numeric expansions of T lymphocytes [5–7]. At the end of the ex vivo culture, both TIL and antigen-specific CTL show potent effector function, but they remain highly dependent from exogenous cytokines for their continuous growth. Unfortunately, γc cytokines, such as IL-2 and IL-15, are usually unavailable at the tumor site at the doses used in culture conditions, so that transferred TIL and CTL are rapidly starved from these crucial survival factors in vivo.

Several strategies have been explored to sustain the in vivo proliferation and persistence of adoptively transferred TIL and CTL. One approach relies on the administration of the recombinant IL-2. For a long time, this cytokine, which mediates its effects through the binding with a heterotrimeric receptor complex composed of the IL-2Rα, IL-2Rβ, and common γ-chain (γc) [8], has been considered the ideal T-cell growth factor despite the fact that it causes an array of adverse effects resulting from the capillary leak syndrome and lymphoid infiltration in many organs [9, 10]. More recently however, it has become evident that because of the broad expression of its receptor, IL-2 can also promote the expansion of regulatory T cells, a subset of T cells that impairs the function of antigen-specific T lymphocytes, including TIL and CTL [11]. Thus, evidences in the field of adoptive immunotherapy are accumulating suggesting that IL-2 may not be the most favorable γc cytokine to sustain the expansion and persistence of adoptively transferred TIL and CTL in vivo.

IL-7 and IL-15 are other crucial homeostatic cytokines, and the recombinant forms of these proteins have been recently introduced in the clinical arena. IL-7 seems well tolerated in preliminary clinical studies and supports the proliferation of both CD4 and CD8, naïve and memory T cells, and immature B cells [12]. However, the utility of this cytokine in supporting adoptively transferred T cells is questionable since a key component of its receptor, the alpha subunit (IL-7Rα or CD127), is usually downregulated in TIL and CTL expanded ex vivo, so that these cells are impaired in their capacity to use either the IL-7 physiologically available in the circulation or in lymphodepleted conditions or the administrated recombinant protein. IL-15 shows promising results in preclinical studies in nonhuman primates, as it can induce the expansion of natural killer cells, CD8 and CD4 from both central-memory and effector-memory compartment [13]. Importantly, even if TIL and CTL do not express the high-affinity receptor for IL-15 (IL-15Rα), IL-15 may still support the growth of TIL and CTL as the recombinant cytokine can simply function by engaging the β and γc chains of the IL-2 receptor complex. However, it remains to be defined whether IL-15 is safe in human subjects or if it shares some of the toxic effects observed with IL-2.

Considering the limitations associated with the systemic administration of γc cytokines, several strategies have been

exploited to reduce the toxicity and the off-target effects of these cytokines. The genetic manipulation of T lymphocytes offers the unique opportunity to restore the capacity of these cells to produce their own cytokines or to express cytokine receptors aimed at creating responsiveness to homeostatic cytokines or cytokines aberrantly expressed by tumor cells. The transgenic expression of cytokines such as IL-2 and IL-15 by ex vivo-expanded antigen-specific T cells has been extensively evaluated in preclinical models and reached the clinical application [14–17].

This chapter focuses on an alternative genetic approach: the ectopic expression of cytokine receptors or growth factor receptors in antigen-specific T cells. This effect is accomplished through the expression of chimeric cytokine receptors (CCR) that are fusion proteins in which the signaling domains of the β and γc chains of the IL-2 receptor complex are fused with the extracellular portion of receptors for cytokines that physiologically do not support the growth of T cells. For example, the extracellular portions of the granulocyte–macrophage colony-stimulating factor (GM-CSF) and erythropoietin (Epo) have been fused with IL-2βγc to create GM-CSF/IL-2βγc [18] and Epo/IL-2βγc [19] chimeric receptors, respectively. T cells expressing GM-CSF/IL-2βγc become therefore responsive to GM-CSF, locally produced by activated T cells or administered as a recombinant protein. Similarly, T cells expressing the Epo/IL-2βγc become responsive to Epo, which can be administered as a recombinant protein. CCR can similarly be used to redirect the responsiveness of T cells toward inhibitory cytokines, thus converting a negative signal into a positive signal. An example of this strategy is represented by the fusion IL-4Rα/IL-2βγc chimeric receptor to combine the extracellular domain of the IL-4 receptor with the signaling domains of IL-2βγc [20, 21]. As a result, these genetically modified T cells proliferate in response to the inhibitory cytokine IL-4, often released within the tumor microenvironment. Although very promising in preclinical models, these approaches may have significant limitations in cancer patients. For example, the release of GM-CSF by activated T cells may be insufficient to support their own growth. Similarly, IL-4 is detected at significant levels (superimposable to that achieved in vitro cultures) only in patients with advanced stage tumors who usually do not benefit from T-cell-based therapies. Finally, the doses of recombinant GM-CSF and Epo required in vivo to support the growth of T cells may induce significant expansion of cell subsets physiologically responding to these growth factors and thus induce significant side effects.

The approach that we describe here is based on the constitutive expression of IL-7Rα—the key receptor for IL-7—by antigen-specific CTL [22]. IL-7 is a nonredundant cytokine involved in primary T- and B-cell development. It is produced by stromal cells, keratinocytes, and gut epithelial cells and is continuously

available in secondary lymphoid organs to support the survival of naïve T cells and memory cells. Furthermore, IL-7 levels in the plasma are inversely correlated with the number of T cells, so that IL-7 is the main cytokine driving the immune reconstitution in lymphopenic conditions [23]. Several studies have shown that CTL and TIL used for adoptive cell therapy lack the expression of IL-7Rα and thus do not respond to IL-7. Restoring the expression of IL-7Rα by these cells, through gene transfer, results in their proliferation in response to IL-7 without modifications of their antigen specificity. This proposed strategy appears advantageous as compared to other approaches since it restores the responsiveness of ex vivo-expanded CTL and TIL to a cytokine that physiologically controls the homeostasis of T cells, allowing their favorable expansion in lymphodepleted hosts and upon administration of recombinant IL-7 that was well tolerated in phase I clinical studies [12].

In this chapter, we describe the protocol optimized to efficiently transduce antigen-specific CTL with a retroviral vector encoding the IL-7Rα, to selectively expand them, and to test their function.

2 Materials

2.1 Genetic Modification of EBV-Specific CTL (EBV-CTL)

1. Peripheral blood mononuclear cells (PBMC) from EBV-seropositive donors.
2. EBV-immortalized lymphoblastoid cell lines (EBV-LCL), cultured in Roswell Park Memorial Institute (RPMI)-1640 medium, supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/mL of penicillin, and 100 mg/mL of streptomycin.
3. CTL medium: 45 % RPMI-1640 medium, 45 % Click's Medium supplemented with 10 % FBS, glutamine, and penicillin–streptomycin.
4. Recombinant human (rh) IL-2. Reconstitute in medium at 200 U/mL. Store at −80 °C. A single aliquot can be used up to 5×.
5. rhIL-7: Reconstitute in medium at 10 ng/mL. Store at −80 °C. A single aliquot can be used up to 5×.
6. 24-well non-tissue culture-treated plates.
7. 24-well tissue culture-treated plates.
8. Recombinant fibronectin fragment: RetroNectin (FN CH-296) (Takara Shuzo, Otsu, Japan). Reconstitute in water at 1 mg/mL and store aliquots at −20 °C. A single aliquot can be used up to 3×.

9. IL-7Rα retroviral supernatant (Vector Production Facility, Baylor College of Medicine, Houston, TX). Store at –80 °C. Do not refreeze.
10. Enzyme-free cell dissociation solution.
11. Trypan blue.

2.2 Immunophenotyping

1. Monoclonal antibodies conjugated with different fluorochromes: CD3-APC, CD4-PERCP, CD8-APC, CD127-PE.
2. 5-mL polystyrene tubes.
3. FACS wash buffer: 1 % FBS in phosphate-buffered saline (PBS).
4. FACSCalibur system equipped with the filter set for quadruple fluorescence signals and CellQuest software.

2.3 STAT5 Phosphorylation

1. 5-mL polystyrene tubes.
2. rhIL-2.
3. rhIL-7.
4. FACS buffer.
5. Fixation buffer: 2 % paraformaldehyde in PBS.
6. Permeabilization buffer: 90 % methanol in water.
7. Alexa Fluor 647 mouse anti-STAT5 (pY694) (BD Phosflow Reagents, San Jose, CA, USA).

2.4 Cytotoxicity Assay

1. 100 μC ^{51}Chromium (MP Biomedicals, Solon, OH, USA).
2. 96-well V-bottom plates.
3. Triton-X: Dilute in water to a final concentration of 1 % and store at room temperature.
4. Packard Cobra Quantum gamma counter (Packard Instrument Company, Downers Grove, IL, USA).
5. Target cells: Autologous LCL, HLA-mismatched LCL (negative control to exclude alloreactivity), and K562 (negative control to exclude NK activity). These cells are maintained in culture in T75 flasks in RPMI-1640, supplemented with 10 % FBS, glutamine, penicillin, and streptomycin at 37 °C, 5 % CO_2.

2.5 Coculture Assay

1. CTL medium.
2. rhIL-2.
3. rhIL-7.
4. 24-well tissue culture-treated plates.

3 Methods

3.1 Genetic Modification of EBV-CTL

Here, we describe the generation of EBV-CTL and their transduction with a retroviral vector.

1. Thaw PBMC, wash, count, and resuspend at 2×10^6/mL in complete medium. Irradiate autologous EBV-LCL at 40 Gy, wash, count, and resuspend at 5×10^4 cell/mL in complete medium. Incubate in 24-well plates at 1 mL/well of PBMC and 1 mL/well of irradiated EBV-LCL (40:1 effector to target ratio).
2. After 10–12 days, collect EBV-CTL, wash, count, and resuspend at 1×10^6/mL in complete medium. Irradiate autologous EBV-LCL at 40 Gy, wash, count, and resuspend at 2.5×10^5 cell/mL in complete CTL medium. In a 24-well plate, cocultivate 1 mL/well of PBMC and 1 mL/well of irradiated EBV-LCL (4:1 effector to target ratio).
3. After 3–4 days, remove 1 mL of medium and replace with complete medium containing 50 U/mL of IL-2.
4. After 3 days, collect CTL, wash, count, resuspend them at 1×10^6 cells/mL, and aliquot 1 mL/well in 24-well tissue culture plate; add 1 mL of irradiated autologous EBV-LCL (40 Gy) resuspended at 2.5×10^5 cell/mL in complete medium containing 50 U/mL of IL-2.
5. On day 3, coat the required number of wells of a non-tissue culture-treated 24-well plate with RetroNectin at a concentration of 7 μg/mL of PBS/well and incubate at 4 °C for 16–24 h.
6. On day 4, remove the RetroNectin-coated plate from 4 °C, aspirate RetroNectin, and wash the plate with 1 mL of complete medium for 10–30 min.
7. Add 0.5 mL/well of the retroviral supernatant and incubate for 20 min in the biosafety cabinet. Aspirate and add another 0.5 mL of retroviral supernatant for 20 min (*see* **Note 1**).
8. Aspirate and add 1.5 mL of retroviral supernatant and 0.5 mL of T cells resuspended at the concentration of 1×10^6/mL in complete medium containing 100 IU/mL rhIL-2 (*see* **Note 2**).
9. Spin plate at $1{,}000 \times g$ for 30 min and incubate at 37 °C for 72 h.
10. After 72 h of incubation, harvest the cells from each well and remove eventual adherent cells by using 0.5 mL cell dissociation medium per well, 5 min incubation and restimulate 1×/week for 2 weeks with irradiated (40 Gy) autologous EBV-LCL (4:1 effector to target ratio), with the addition of 2.5 ng/mL of exogenous rhIL-7 or 50 U/mL of rhIL-2 (*see* **Note 3**).
11. Assess transduction efficiency of EBV-CTL by FACS analysis using CD127-PE antibody (*see* Subheading 3.2).

3.2 Testing the Transduction Efficiency

Expression of the CD127, assessed by FACS analysis, is used to evaluate the transduction efficiency of EBV-CTL.

1. Collect control non-transduced (NT) and IL-7Rα⁺ EBV-CTL, wash, count them, and resuspend at the concentration of 1×10^6/mL in FACS wash buffer.
2. Aliquot 0.2 mL of cells in polystyrene tubes.
3. Add 1 μL of CD127-PE to each tube.
4. Optional: Add 1 μL of other monoclonal antibodies, like differently conjugated CD3 and CD4 and CD8 to assess the expression of CD127 by the different T cells.
5. Incubate at 4 °C, in the dark for 30 min.
6. Wash with 2 mL of FACS buffer, spin, and decant. Resuspend in 0.2 mL of FACS buffer.
7. Assess the transduction efficiency at the FACS as shown in Fig. 1.

3.3 Testing Functionality of the IL-7Rα

3.3.1 Expansion of IL-7Rα⁺ EBV-CTL Is Determined by Cell Count of Viable Cells

1. Collect and count EBV-CTL using a 1:1 dilution with Trypan blue to determine viability of CTL; resuspend CTL in CTL medium at the concentration of 1×10^6/mL and aliquot at 1 mL/well.
2. Add 1 mL of irradiated autologous EBV-LCL (40 Gy) resuspended at 2.5×10^5/well in complete medium with 2.5 ng/mL of exogenous rhIL-7.
3. Incubate at 37 °C, 5 % CO_2.
4. On day 4, remove 1 mL of medium and replace with fresh medium, with the addition of 2.5 ng/mL of exogenous rhIL-7.
5. Incubate at 37 °C, 5 % CO_2.
6. On day 7, collect and count EBV-CTL using Trypan blue to determine their viability.
7. Repeat weekly stimulation, from **steps 1** to **6**.

3.3.2 STAT5 Phosphorylation Is Used to Assess the Signaling of the Receptor

1. Collect NT and IL-7Rα⁺ EBV-CTL, wash, count them, and resuspend at the concentration of 1×10^6/mL in complete medium. Aliquot cells in 5-mL polystyrene tubes to a concentration of 1×10^6/tube.
2. Add 2.5 ng/mL of exogenous rhIL-7 or 50 U/mL of rhIL-2.
3. Incubate for 15 min at 37 °C, 5 % CO_2.
4. Wash with 2 mL of FACS buffer, spin, and decant.
5. Add 1 mL of the fixation buffer, incubate for 10 min at 37 °C, spin, and decant.
6. Add 1 mL of permeabilization buffer, incubate for 30 min on ice, spin, and decant.

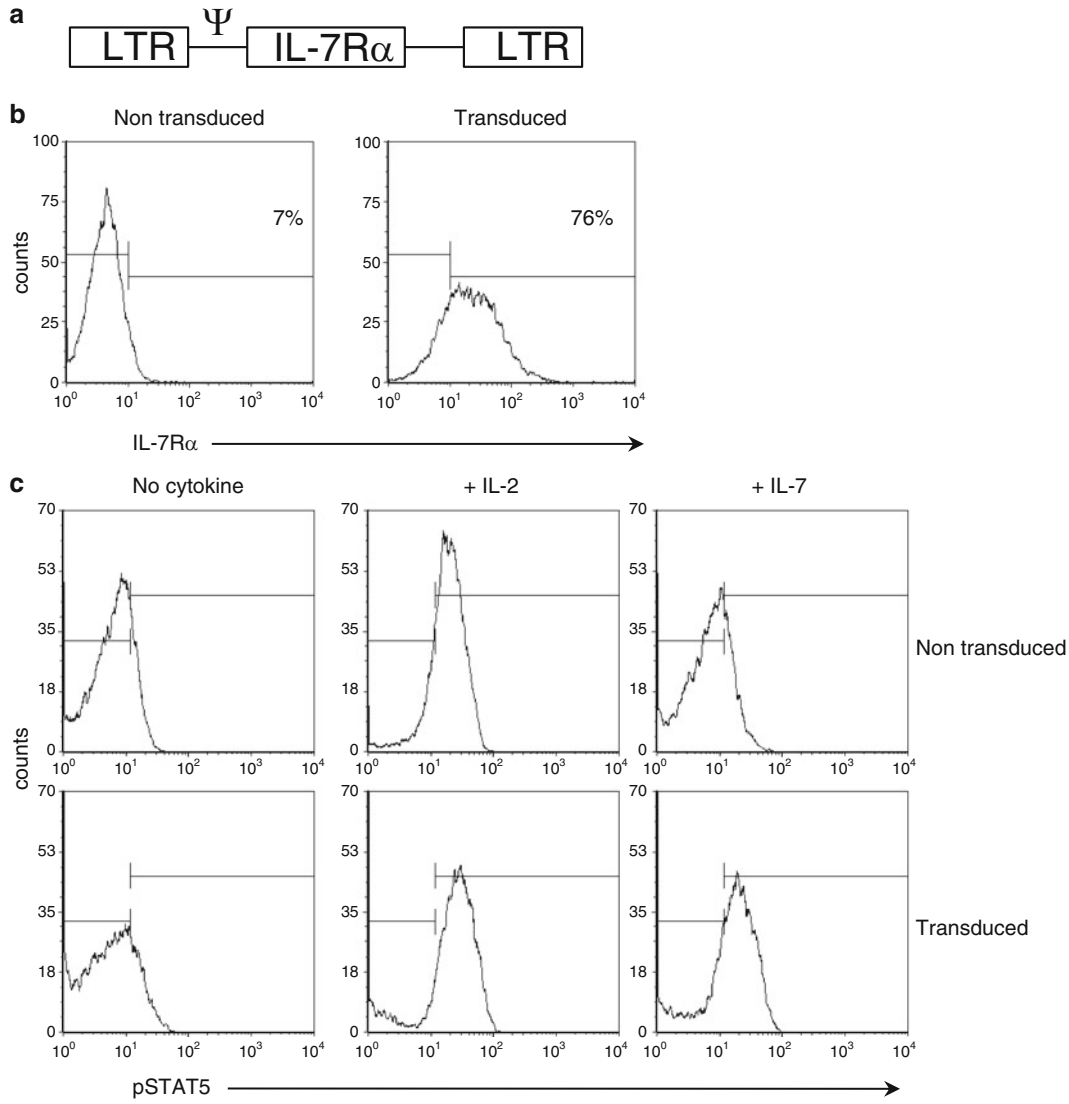

Fig. 1 Transduction efficiency of genetically modified EBV-CTL and assessment of the functionality of the IL-7Rα transgene. Panel (**a**) shows the schema of the gamma-retroviral vector used to transduce the EBV-CTL. Panel (**b**) shows the transduction efficiency of one representative EBV-CTL line. CTLs were transduced with the IL-7Rα vector and the expression of the transgene was measured on the cell surface by FACS analysis. Panel (**c**) shows the phosphorylation of STAT5 in response to IL-2 or IL-7 in control non-transduced (NT) and transduced EBV-CTL. STAT5 is phosphorylated in both NT and transduced EBV-CTL in response to IL-2, but it is phosphorylated only in IL-7Rα$^+$ EBV-CTL in response to IL-7

7. Wash with FACS buffer 2×.
8. Add 10 μL of Alexa Fluor 647 mouse anti-STAT5, and incubate for 1 h at room temperature, in the dark.
9. Wash and proceed with the FACS acquisition.

3.3.3 Short-Term Cytotoxicity Assay

This short-term assay is used to ensure that EBV-CTL retain their ability to lyse their native target cells.

1. Collect and pellet at least 5×10^6 target cells (*see* **Notes 4** and **5**).
2. Resuspend target cells by finger-clicking and perform radiolabeling by adding 100 μCi of ^{51}Chromium in a radioactive safety cabinet. Labeled cells are then incubated for 1 h at 37 °C, with a gentle finger flicking every 15 min.
3. Wash ^{51}Chromium-labeled target cells by centrifugation at $400 \times g$ for 3 min using 5 mL of complete medium; count cells after the fourth wash, and then resuspend them at the concentration of 5×10^4/mL in complete medium.
4. Collect transduced EBV-CTL cultivated with rhIL-2 or rhIL-7, wash, count them, and resuspend at the concentration of 2×10^6/mL in complete medium. Aliquot cells in a 96-well V-bottom plate. Perform serial dilutions (1:2) to obtain triplicates of wells containing cell numbers ranging from 2×10^5/well to 2.5×10^4/well.
5. Add 100 μL of the appropriate target cells to the wells containing 100 μL of the diluted EBV-CTL, 100 μL of medium only (spontaneous release), or 100 μL of 1 % Triton-X (maximum release).
6. Incubate the plates for 4–5 h at 37 °C, 5 % CO_2.
7. Spin plate at $400 \times g$ for 3 min, collect 100 μL of supernatant, transfer in appropriate tubes, and read using the γ-counter (*see* **Note 6**).
8. The percent of killing is calculated as follows:
   [cpm from experimental wells (target + CTL) – cpm of target cells in the presence of medium only (spontaneous release)/cpm of target cells in the presence of 1 % Triton-X (maximum release) – cpm of target cells in the presence of medium only (spontaneous release)] × 100.

3.3.4 Coculture Assay

This assay is used to ensure that EBV-CTL retain their ability to eliminate from the culture their native target cells in a long-term assay.

1. Collect autologous EBV-LCL, wash, count them, and resuspend at the concentration of 2×10^6/mL in complete medium. Aliquot cells in a 24-well tissue culture-treated plate to a concentration of 1×10^6/well.
2. Collect NT and IL-7Rα$^+$ EBV-CTL, wash, count them, and resuspend at the concentration of 0.5×10^6/mL in complete medium. Aliquot cells in a 24-well tissue culture-treated plate to a concentration of 0.5×10^6/well.
3. Add 2.5 ng/mL of exogenous rhIL-7 or 50 U/mL of rhIL-2.

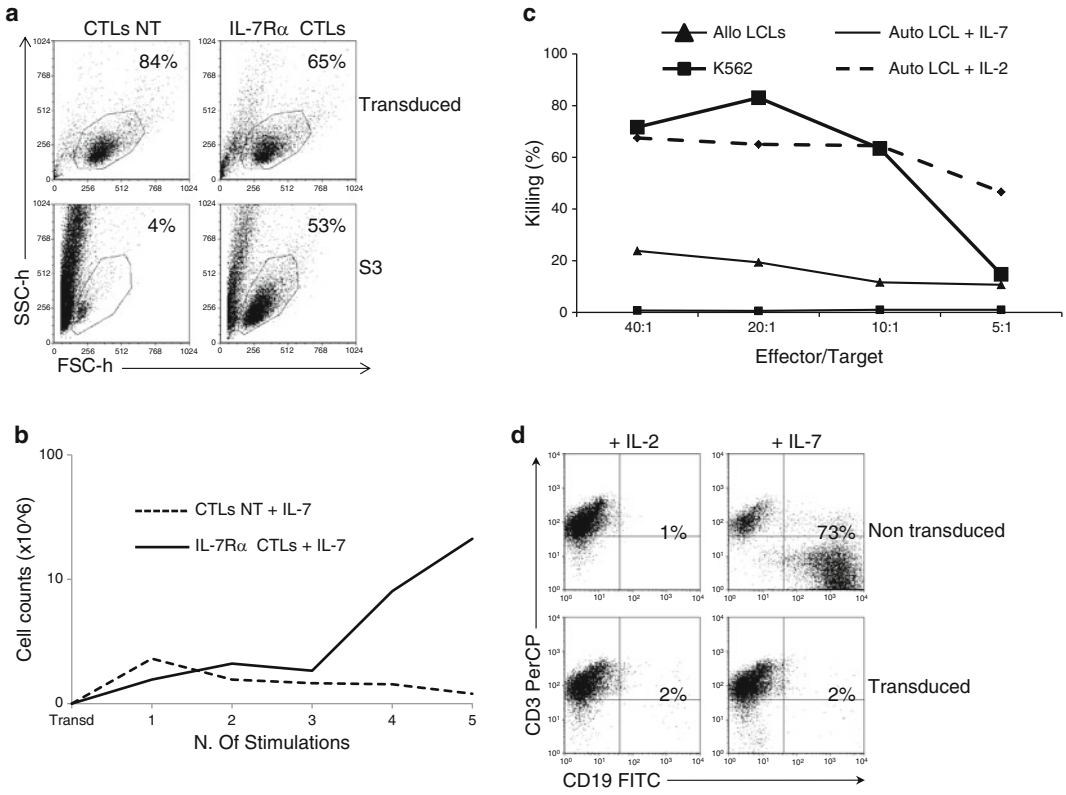

Fig. 2 Functional characterization of genetically modified EBV-CTL. Panel (**a**) shows the viable gating assessing the physical parameters by FACS analysis in control NT and IL-7Rα$^+$ EBV-CTL. EBV-CTL viability was assessed immediately after the transduction and after 3 weeks of culture in which EBV-CTL were weekly stimulated with the antigen and in the presence of IL-7. NT EBV-CTL do not survive in culture since they do not respond to IL-7, while IL-7Rα$^+$ EBV-CTL remain viable when stimulated with the antigen in the presence of IL-7. Panel (**b**) shows that IL-7Rα$^+$ EBV-CTL but not control NT cells expand in vitro when stimulated weekly with autologous EBV-LCL in the presence of IL-7. Panel (**c**) shows the results of a representative ^{51}Chromium-release assay. IL-7Rα$^+$ EBV-CTL induce comparable lysis of the autologous EBV-LCL when cultivated in IL-2 or IL-7, confirming retained killing activity against the native target antigen. The lysis of allogeneic EBV-LCL, regardless of whether CTL are grown in IL-2 or IL-7, is negligible, confirming retained MHC restriction. No significant reactivity (<10 %) is observed against the K562 cell lines. Panel (**d**) shows the plot of a representative coculture experiment of EBV-CTL and autologous EBV-LCL. The ratio EBV-CTL to EBV-LCL was 1:2. Residual EBV-LCL were quantified by day 7 of culture based on their CD19 expression by FACS analysis. Control NT and IL-7Rα$^+$ EBV-CTL eliminated equally well EBV-LCL in the presence of IL-2. By contrast, only IL-7Rα$^+$ EBV-CTL eliminated EBV-LCL in the presence of IL-7

4. Incubate for 7 days at 37 °C, 5 % CO_2.
5. Assess cytotoxic activity of NT or IL-7Rα$^+$ EBV-CTL by FACS analysis using anti-CD3-PerCP and anti-CD19-FITC antibodies as shown in Fig. 2.

4 Notes

1. Avoid multiple thawing and freezing of the supernatants.
2. Keep some non-transduced (NT) CTL as controls. These cells will need to be plated at the same concentration in complete medium containing 2.5 ng/mL of rhIL-7 in a 24-well tissue culture-treated plate.
3. The cell count of the transduced EBV-CTL is expected to drop during the first 2 weeks in culture in the presence of rhIL-7 due to the selection of the IL-7Rα$^+$ EBV-CTL.
4. Do not provide the culture with IL-7 for at least 3 days before testing the expression of IL-7Rα to avoid significant underestimation of the receptor expression.
5. To evaluate EBV-CTL specificity, autologous EBV-LCL, HLA class I and II mismatched EBV-LCL, and K562 cell lines are used as target cells. If CTL are antigen specific, only autologous EBV-LCL are expected to be significantly lysed.
6. Wear appropriate radioprotection equipment and monitor the work area using a survey meter. Label and dispose of radioactive waste according to approved guidelines. Personnel monitoring with a thermoluminescence dosimeter is recommended.

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Highlights of This Issue

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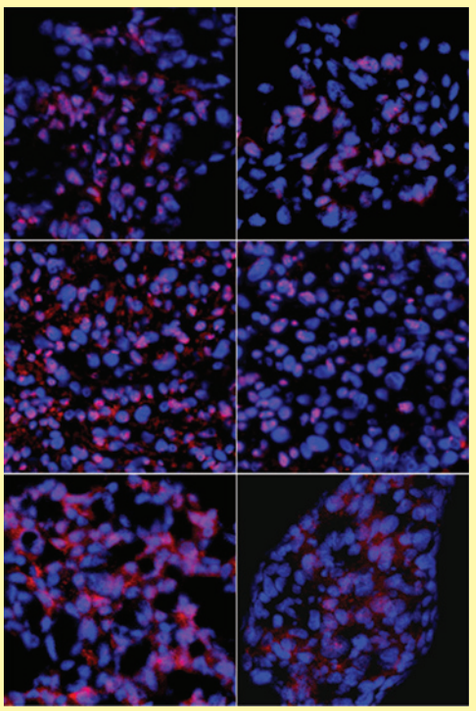

Phase I Study of SAR245408 in Patients with Solid Tumors

Shapiro *et al.* **Page 233**

Aberrant activation of the phosphatidylinositol-3-kinase (PI3K) pathway contributes to tumor cell growth, proliferation and survival, and may confer resistance to chemotherapy and targeted agents. Shapiro and colleagues have conducted a Phase 1 trial of SAR245408 (XL147), an orally bioavailable pan-class I PI3K inhibitor. Pathway inhibition in tumor and surrogate tissues and preliminary clinical activity were observed at tolerable doses, irrespective of tumor PI3K pathway molecular alterations. The results provide the groundwork for future monotherapy and combination studies.

CD137 Expression Identifies Tumor-Reactive TILs

Ye *et al.* **Page 44**

The association between of increased intratumoral T-cell accumulation and survival suggests the existence of naturally occurring tumor-reactive T-cells in human cancer. However, the immunobiology of spontaneous tumor-reactive T-cells in cancer is not well defined because identifying and validating these responses is difficult. Ye and colleagues elucidated CD137 as a biomarker for naturally occurring tumor-reactive T-cells in cancer and developed a rapid, accurate system to comprehensively isolate TILs with tumor-rejecting capability directly from resected human tumors. Thus, CD137 plays an important role in the immunobiology of human cancer, rationalizing its agonistic engagement *in vivo* and its use in TIL selection for adoptive immunotherapy trials.

IL7 Overcomes Treg Inhbition of CAR-Modified CTLs

Perna *et al.* **Page 131**

Adoptive transfer of virus-specific CTLs expressing a chimeric antigen receptor (CAR) represents a promising immunotherapy approach. However, regulatory T cells (Tregs) that are abundant within the tumor environment impair proliferation and function of CAR-redirected CTLs. Perna and colleagues exploited a modification of the IL-7/IL-7Rα axis in CAR-redirected CTLs to counter Treg inhibition. The authors found that IL-7, unlike IL-2, supports the antitumor activity of EBV-CTLs genetically manipulated to coexpress IL-7Rα and a GD2-specific CAR both *in vitro* and *in vivo* in the presence of Tregs. Thus, the proposed genetic modification may further improve the clinical outcome of CAR-modified CTLs.

Transient Hh Activation Rescued IR-Induced Hyposalivation

Hai *et al.* **Page 140**

Irreversible hyposalivation is common in head and neck cancer survivors treated with radiotherapy, for which no curative treatment is available currently. Hai and colleagues found that the Hedgehog signaling pathway in salivary glands was activated during functional regeneration after duct ligation but not activated after irradiation, and the transient activation of this pathway after irradiation rescued salivary gland hypofunction by preserving salivary stem/progenitor cells and the parasympathetic innervation in mice. Similar effects on expression of genes essential for these two aspects were observed in cultured human salivary epithelial cells, suggesting the potential of this strategy in treating radiotherapy-induced hyposalivation.

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Clinical Cancer Research

Interleukin-7 Mediates Selective Expansion of Tumor-redirected Cytotoxic T Lymphocytes (CTLs) without Enhancement of Regulatory T-cell Inhibition

Serena K. Perna, Daria Pagliara, Aruna Mahendravada, et al.

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Cancer Therapy: Preclinical

Clinical Cancer Research

Interleukin-7 Mediates Selective Expansion of Tumor-redirected Cytotoxic T Lymphocytes (CTLs) without Enhancement of Regulatory T-cell Inhibition

Serena K. Perna[1], Daria Pagliara[1,5], Aruna Mahendravada[1], Hao Liu[1], Malcolm K. Brenner[1,2,4], Barbara Savoldo[1,2], and Gianpietro Dotti[1,3,4]

Abstract

Purpose: The antitumor activity of chimeric antigen receptor (CAR)–redirected CTLs should be enhanced if it were possible to increase their proliferation and function after adoptive transfer without concomitantly increasing the proliferation and function of regulatory T cells (Treg). Here, we explored whether the lack of IL-7Rα in Treg can be exploited by the targeted manipulation of the interleukin-7 (IL-7) cytokine–cytokine receptor axis in CAR-engrafted Epstein–Barr Virus–specific CTLs (EBV-CTLs) to selectively augment their growth and antitumor activity even in the presence of Treg.

Experimental Design: We generated a bicistronic retroviral vector encoding a GD2-specific CAR and the IL-7Rα subunit, expressed the genes in EBV-CTLs, and assessed their capacity to control tumor growth in the presence of Treg *in vitro* and *in vivo* when exposed to either interleukin-2 (IL-2) or IL-7 in a neuroblastoma xenograft.

Results: We found that IL-7, in sharp contrast with IL-2, supports the proliferation and antitumor activity of IL-7Rα.CAR-GD2$^+$ EBV-CTLs both *in vitro* and *in vivo* even in the presence of fully functional Treg.

Conclusions: IL-7 selectively favors the survival, proliferation, and effector function of IL-7Rα-transgenic/CAR-redirected EBV-CTLs in the presence of Treg both *in vitro* and *in vivo*. Thus, IL-7 can have a significant impact in sustaining expansion and persistence of adoptively CAR-redirected CTLs. *Clin Cancer Res; 20(1); 131–9. ©2013 AACR.*

Introduction

The expression of chimeric antigen receptors (CAR) in T lymphocytes to redirect their antigen specificity has significantly expanded the clinical application of adoptive T-cell immunotherapies against a variety of human malignancies (1, 2). CAR molecules are chimeric proteins, in which a single chain antibody-binding site is fused with the signaling domain CD3ζ that activates T lymphocytes upon binding to the tumor antigen (3). However, in this form, CAR molecules do not provide adequate costimulation to T cells (1, 4, 5). To overcome this limitation, CARs can be expressed by CTLs whose native receptors are specific for virus latency proteins such as those derived from the Epstein–Barr Virus–specific CTLs (EBV-CTLs; refs. 6, 7). These virus-specific CTLs can receive physiologic costimulation from professional antigen presenting cells processing latent viral antigens and kill tumor cells through their CAR (6, 7). Although this approach can produce complete and sustained antitumor responses, for example in some patients with neuroblastoma, in most recipients, CAR-engrafted EBV-CTLs have limited *in vivo* survival and fail to consistently eradicate disease (8, 9). It is likely that the combination of host/tumor associated inhibitory factors and insufficient *in vivo* immunostimulation limit the expansion and persistence of these cells (10).

Regulatory T cells (Treg) play a significant role in impairing the antitumor effects of tumor-specific CTLs (11). Treg are frequently increased in the peripheral blood and in tumor biopsies of patients with cancer (12–17) and their presence often correlates with poor clinical outcome (15). Thus, the development of strategies aimed at eliminating Treg or at selectively favoring the expansion of antitumor CTLs may significantly contribute in enhancing the engraftment and antitumor effects of adoptively transferred CTLs. To date, most efforts to increase *in vivo* immunostimulation of adoptively transferred T cells have focused on administration of interleukin (IL)-2 (18). Although this cytokine is a potent T-cell growth factor, it is not selective for effector T-cell subsets and can also enhance the growth and inhibitory activity of Treg (19).

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Translational Relevance

Adoptive transfer of virus-specific CTLs expressing a chimeric antigen receptor (CAR) represents a promising therapy for patients with cancer. However, the *in vivo* expansion of these cells remains suboptimal so that new strategies are required to selectively expand them without favoring the concomitant proliferation and function of regulatory T cells (Treg) that are often abundant in patients with cancer. Our study provides preclinical data, indicating that the manipulation of the interleukin (IL)-7 cytokine–cytokine receptor axis in CAR-engrafted Epstein–Barr Virus–specific CTLs (EBV-CTLs) can be used to selectively expand the CTLs while avoiding the inhibitory effects of Treg, which would otherwise be enhanced by use of the more broadly acting T-cell growth factor IL-2.

One means by which T lymphocytes can be selectively expanded is by using IL-7, a γ-chain cytokine that promotes homeostatic expansion of naïve and memory T cells but has no activity on Treg, which lack the IL-7Rα (the private chain of the IL-7 receptor; refs. 20–23). Administration of recombinant IL-7 was well tolerated in early-phase clinical trials, and expanded naive and central-memory T-cell subsets but not Treg (20, 21). Unfortunately, under physiologic conditions, IL-7 cannot support the *in vivo* expansion of adoptively transferred CAR-redirected CTLs as this is an effector-memory T-cell subset that, like Treg, also lacks IL-7Rα (24).

Here, we developed models *in vitro* and *in vivo* to demonstrate that human Treg clearly inhibit the antitumor effects of CAR-redirected EBV-CTLs. We also show that selective modulation of the IL-7 cytokine–cytokine receptor axis in CAR-engrafted EBV-CTLs augments their antitumor effects *in vivo* in the presence of Treg. This strategy should safely enhance the persistence and survival of adoptively transferred CAR-redirected virus-specific CTLs in patients with cancer.

Materials and Methods

Plasmid construction, retrovirus production, and tumor cell lines

The full-length human IL-7Rα linked through the 2A (TAV) sequence to the CAR-GD2 encoding the CD28 endodomain (25) was cloned into the SFG retroviral vector to generate the bicistronic vector SFG.IL-7Rα.CAR-GD2. The retroviral vectors encoding eGFP and Firefly Luciferase (FFLuc) were previously described (26). Retroviral supernatant was prepared using transient transfection of 293T cells (26). The neuroblastoma cell line CHLA-255 (ref. 27; kindly provided by Dr. Leonid Metelitsa, Texas Children's Hospital, Baylor College of Medicine, Houston, TX) was derived from a patient, and we verified that this line retains the surface expression of the target antigen GD2.

Generation and transduction of EBV-CTLs

EBV-transformed lymphoblastoid cells (LCL) and EBV-CTLs were prepared using peripheral blood mononuclear cells (PBMC), obtained from healthy donors as previously described (28). EBV-CTLs were transduced with retroviral supernatant after three stimulations with autologous LCLs, as previously described (8), and then maintained in culture by weekly stimulation with LCLs and recombinant IL-2 (50 IU/mL) or IL-7 (2.5 ng/mL; PeproTech).

Expansion of Treg

To obtain significant numbers of cells for the *in vitro* and *in vivo* experiments, Treg were isolated and expanded as previously described (29). Briefly, $CD25^{bright}$ T cells were purified from PBMCs by positive selection using immunomagnetic selection in the presence of nonsaturating concentrations (2 μL/1 × 10^7 PBMCs) of anti-human CD25 magnetic beads (Miltenyi Biotech). On day 0, the purified $CD25^+$ T cells were activated in 24-well plates coated with OKT3 (1 μg/mL) and anti-CD28 antibody (BD Pharmingen; 1 μg/mL) in RPMI 1640 in the presence of rapamycin (Sigma) at a final concentration of 100 nmol/L. On days 7 and 14, cells were restimulated with OKT3/CD28 antibodies, irradiated feeder cells, rapamycin, and IL-2 (50 IU/mL) in small bioreactors (G-REX; ref. 29). At the end of the 3-week culture (day 21), cells were used for *in vitro* and *in vivo* experiments. The cell fraction obtained from buffy coats after the selection of $CD25^{bright}$ T cells was further enriched for $CD4^+$ cells which were then used as negative control in parallel culture experiments, in which we evaluated the immunosuppressive activity of Treg (29, 30).

Immunophenotyping

Cells were stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, peridinin-chlorophyll-protein complex (PerCP)-, or allophycocyanin (APC)-conjugated monoclonal antibodies (mAb). We used CD3, CD4, CD8, CD25, and CD127 (IL-7Rα specific) from Becton Dickinson (BD Bioscience) and FoxP3 from eBioscience Inc. CAR-GD2 expression by transduced EBV-CTLs was detected using the specific anti-idiotype antibody 1A7, followed by staining with the secondary antibody RAM-IgG1-PE (Becton Dickinson; ref. 8). STAT5 phosphorylation in Treg and EBV-CTLs was assessed after cytokine stimulation for 15 minutes using the anti-phospho-STAT5 (Y694) mAb-Alexa Fluor 647 Conjugate (BD Phosflow Reagents). Cells were analyzed using a BD FACSCalibur system equipped with the filter set for quadruple fluorescence signals and the CellQuest software (BD Biosciences). For each sample, we analyzed a minimum of 10,000 events.

Carboxyfluorescein diacetate succinimidyl ester–based assays

Proliferation of Treg or EBV-CTLs or activated PBMC was assessed by carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution. Briefly, EBV-CTLs were labeled with 1.5 μmol/L CFSE (Invitrogen) and activated with LCLs (ratio 4:1) with or without IL-2 (12.5 IU/mL) or IL-7 (10 ng/mL). CFSE dilution was measured by flow cytometry after 7 days of culture. A similar protocol was used to evaluate the proliferation of CFSE-labeled Treg post activation with

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OKT3, irradiated feeders, and IL-2 or IL-7. To evaluate the suppressive activity of Treg, CFSE-labeled EBV-CTLs were stimulated with LCLs (ratio 4:1) in the presence of Treg or control $CD4^+CD25^-$ cells (ratio, 1:1; ref. 30), and of IL-2 (12.5 IU/mL) or IL-7 (10 ng/mL). Similarly, PBMC depleted of $CD25^{bright}$ cells were stained with CFSE and activated in the presence of irradiated allogeneic feeders (ratio 2:1) and OKT3 (500 ng/mL; refs. 29, 30). After 7 days, cells were stained with CD8-APC and CD4-PerCP, analyzed by fluorescence-activated cell sorting (FACS) and cell division assessed by CFSE dilution.

Evaluation of antitumor activity

EBV-CTLs were cultured in the presence of the neuroblastoma cell line (CHLA-255) genetically modified to stably express GFP in the presence or in the absence of Treg (at the EBV-CTLs:CHLA-255:Treg ratio of 1:2:1) and of IL-2 (12.5 IU/mL) or IL-7 (5 ng/mL). After 7 days, cells were collected, stained with CD3 to identify T cells, and analyzed by FACS. GFP was used to quantify residual tumor cells in culture.

Xenogenic mouse model

To assess the antitumor effect of EBV-CTLs *in vivo* in the presence of Treg, we used the xenograft mouse model and an *in vivo* imaging system as previously described (7, 24). Mouse experiments were performed in accordance with Baylor College of Medicine's Animal Husbandry guidelines. Briefly, 8- to 10-week-old NOD.Cg-Prkdcscid IL2rgtmWjl/Sz (NSG) mice (Jackson Laboratory, Bar Harbor, ME) were engrafted intraperitoneally with the CHLA-255 cells (1×10^6 cells per mouse) genetically modified with FFluc to monitor tumor growth using the IVIS bioluminescence system (Xenogen IVIS 200 Biophotonic Imaging System). The intraperitoneal model was selected to minimize confounding issues due to suboptimal cell biodistribution and simultaneous colocalization at the tumor site of CAR-modified EBV-CTLs and Treg. When the signal (measured as $p/sec/cm^2/sr$) was consistently increasing, usually by day 7 to 10, mice received intraperitoneal EBV-CTLs (10×10^6 T cells per mouse) with or without Treg (10×10^6 T cells per mouse; two infusions 1-week apart). IL-2 (500 IU/mouse) or IL-7 (200 ng/mouse) were administered intraperitoneally three times a week.

Statistical analysis

All *in vitro* data were summarized by means and SEM. For the bioluminescent experiments, intensity signals were log-transformed and summarized using mean ± SD at baseline and multiple subsequent time points for each group of mice. Changes in intensity of signal from baseline at each time point were calculated and compared using paired *t* tests or Wilcoxon signed-ranks test. When the *P* value was less than 0.05, a mean difference was accepted as statistically significant. For the bioluminescence experiments, intensity signals were log-transformed and summarized using mean and SDs at baseline and multiple subsequent time points for each group of mice. The response profiles over time were analyzed by the generalized estimating equations method for repeated measurements.

Results

Functional IL-7Rα and CAR-GD2 can be coexpressed in EBV-CTLs

To restore the responsiveness to IL-7 and to redirect the antigen specificity of EBV-CTLs against neuroblastoma, we generated a bicistronic γ-retroviral vector encoding the IL-7Rα and a GD2-specific CAR linked through a 2A (TAV) sequence (SFG.IL-7Rα.2A.CAR-GD2; Fig. 1A). EBV-CTLs established from 5 healthy EBV-seropositive donors were transduced with the vector, and the expression of both IL-7Rα and CAR-GD2 was measured by FACS analysis. As shown in Figure 1B, both CAR-GD2 and IL-7Rα were stably expressed (64% ± 3% and 34% ± 9%, respectively) in transduced EBV-CTLs, whereas the expression of the native IL-7Rα on control cells remained negligible (4% ± 1%).

To evaluate the functionality of the transgenic IL-7Rα, we measured the phosphorylation of STAT5 in response to either IL-2 or IL-7. In the absence of cytokines, control and IL-7Rα.CAR-$GD2^+$ EBV-CTLs showed negligible phosphorylation of STAT5 (3% ± 2% and 8% ± 4%, respectively). In IL-7Rα.CAR-$GD2^+$ EBV-CTLs, near equal STAT5 phosphorylation of Tyr-694 was detected in response to IL-2 (49% ± 7%) or IL-7 (38% ± 6%, respectively; P = NS). In contrast, in control cells, STAT5 was phosphorylated in response to IL-2 (63% ± 8%) but not to IL-7 (6% ± 5%; $P < 0.05$; Fig. 1C). The levels of IL-7Rα–dependent STAT5 phosphorylation in IL-7Rα.CAR-$GD2^+$ EBV-CTLs exposed to IL-7 were very similar to the amount observed in T lymphocytes physiologically expressing the IL-7Rα and exposed to IL-7 (Supplementary Fig. S1A). The functionality of the transgenic IL-7Rα was further supported by progressive selection of transgenic cells if cultures were supplemented with IL-7. As illustrated in Figure 1D (and Supplementary Fig. S1B), when IL-7Rα.CAR-$GD2^+$ CTLs were stimulated weekly with autologous LCLs and IL-7, the expression of both IL-7Rα and CAR-GD2 progressively increased between the third and sixth antigen-specific stimulation (from 34% ± 9% to 66% ± 5% for IL-7Rα, and from 64% ± 3% to 80% ± 7% for CAR-GD2). In contrast, when CTLs were expanded in the presence of IL-2, no enrichment of either transgenes was observed, as this cytokine equally supports the *ex vivo* growth of transduced and non transduced CTLs (data not shown).

The enrichment of transgenic T cells following exposure to IL-7 was a consequence of the proliferation of IL-7Rα.CAR-$GD2^+$ EBV-CTLs. As illustrated in Figure 2A, CFSE labeled-control and IL-7Rα.CAR-$GD2^+$ EBV-CTLs divided equally well when stimulated with LCLs (ratio 4:1) in the presence of IL-2 (proliferation, 68% ± 6% and 68% ± 4%, respectively). In contrast, in the presence of IL-7, IL-7Rα.CAR-$GD2^+$ but not control EBV-CTLs had significantly greater proliferation, 63% ± 3% versus 14% ± 1%, respectively ($P < 0.001$). The number of EBV-CTLs proliferating in response to EBV-LCLs and IL-7 was generally higher than expected based on the ectopic expression of IL-7Rα. This higher level is likely a consequence of the physiologic production of IL-2 by EBV-CTLs in response to their cognate EBV antigens (EBV-LCLs; Supplementary Fig. S2). Finally,

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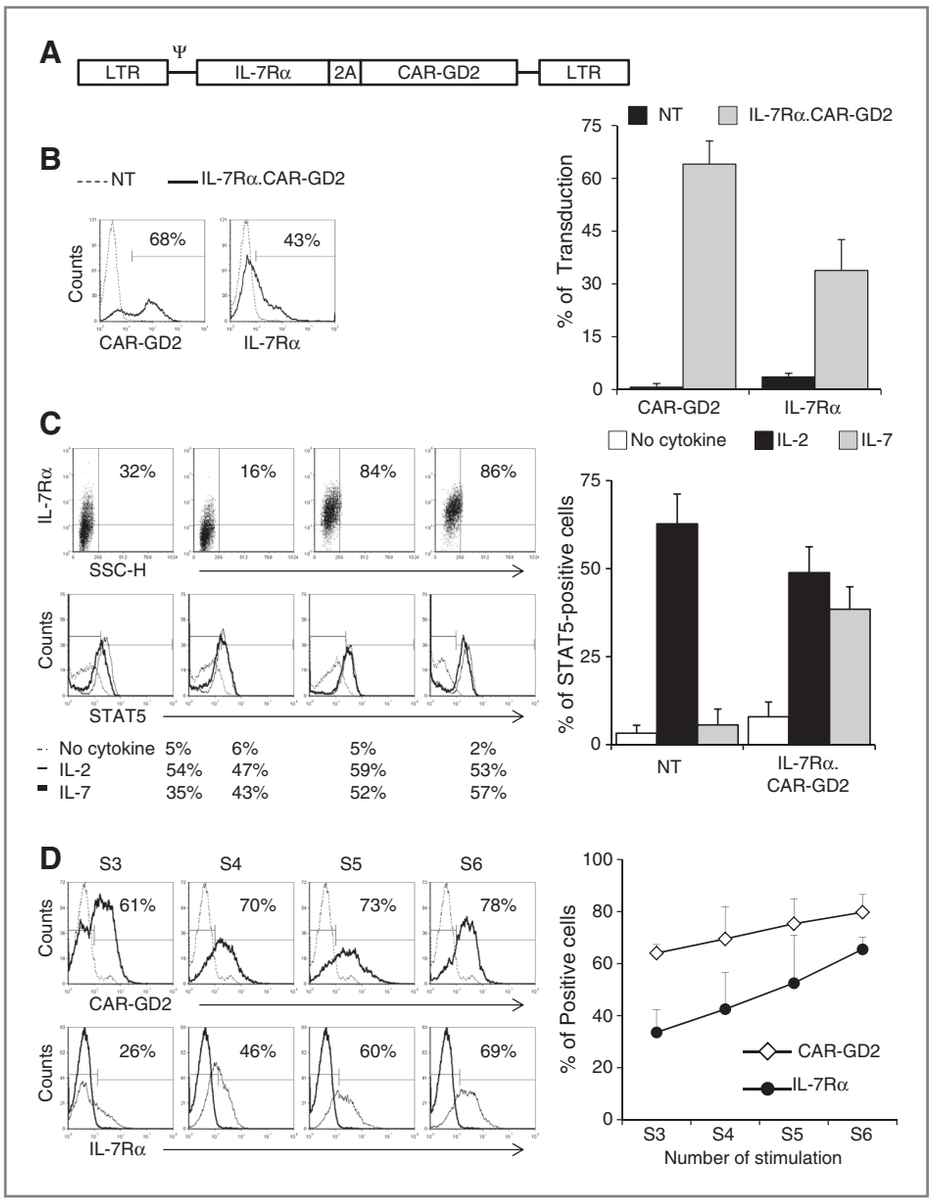

Figure 1. EBV-CTLs are effectively transduced with the bicistronic vector encoding both the IL-7Rα and the CAR-GD2. A, schema of the bicistronic γ-retroviral vector encoding the IL-7Rα and GD2-specific CAR linked through a 2A (TAV) sequence. B, expression of CAR-GD2 (top) and IL-7Rα (bottom) evaluated by FACS analysis day 7 after transduction. The dotted line indicates control EBV-CTLs and the bold line indicates the transduced EBV-CTLs. The graph represents mean ± SD of 5 donors. C, IL-7Rα expression in four IL-7Rα.CAR-GD2$^+$ EBV-CTLs generated (top) and STAT5 phosphorylation (bottom) in the absence of cytokines (thin black line), in response to IL-2 (dotted line), or IL-7 (black bold line). D, progressive enrichment in cells expressing the two transgenes IL-7Rα and CAR-GD2 when IL-7Rα.CAR-GD2$^+$ EBV-CTLs were expanded in the presence of IL-7. S3, S4, S5, and S6 indicate the transgene expression detected week 3 (S3), week 4 (S4), week 5 (S5), and week 6 (S6), respectively after transduction. Graph represents mean ± SEM of four different EBV-CTL lines.

exposure of IL-7Rα.CAR-GD2$^+$ EBV-CTLs to IL-7 did not affect their antitumor properties. As shown in Figure 2B, when EBV-CTLs were cultured with CHLA-255 cells, only IL-7Rα.CAR-GD2$^+$ cells controlled tumor growth in the presence of either IL-2 or IL-7 (6% ± 1% and 4% ± 1%, respectively), whereas tumor cells outgrew in cultures containing control EBV-CTLs irrespective of the cytokine added (43% ± 5% and 57% ± 12%, respectively; $P < 0.001$).

Ex vivo expanded Treg do not respond to IL-7

We used *ex vivo* expanded CD4$^+$CD25$^+$ Treg isolated from healthy donors rather than freshly isolated Treg for the following reasons. First, the experiments required a significant number of Treg that could not be obtained upon fresh isolation even from buffy coat preparations. Second, circulating Treg obtained after immunomagnetic selection on the basis of CD4 and CD25 expression are frequently contaminated by CD4$^+$CD25$^+$IL7Rα$^+$ cells that lack regulatory activity, but respond to IL-7 (data not shown; ref. 31). We first confirmed that the nominal Treg population retained their inhibitory properties. As shown in Figure 3A, the proliferation of activated PBMCs (80% ± 3% in the presence of control CD4$^+$CD25$^-$ cells) was significantly inhibited in the presence of the expanded Treg population (27% ± 6%; $P < 0.001$). We then confirmed that these Treg, like freshly isolated Treg (22), lacked expression of IL-7Rα (3% ± 0.4% positive; Fig. 3B). As a consequence, STAT5 was only phosphorylated in these Treg in response to IL-2 (MFI = 75 ± 9; $P < 0.001$) and not in response to IL-7 (MFI = 23 ± 3; Fig. 3C).

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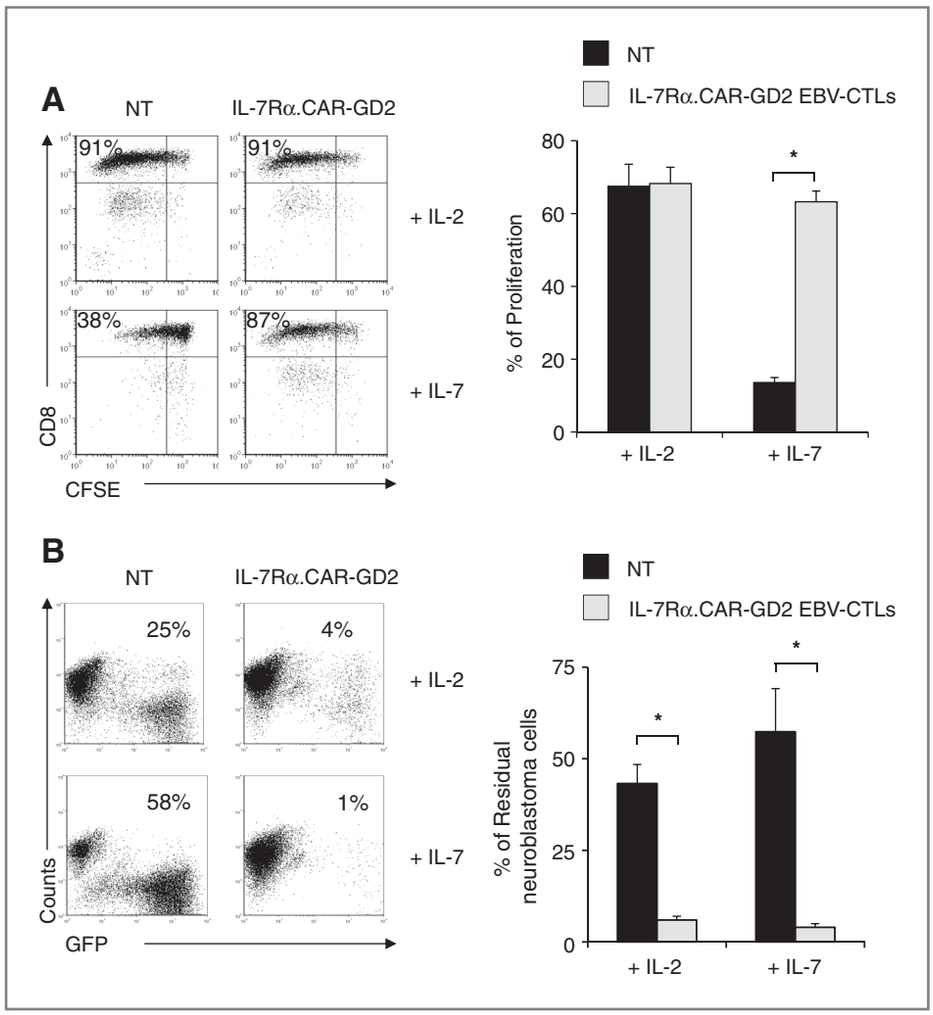

Figure 2. IL-7 supports the proliferation and effector function of IL-7Rα.CAR-GD2$^+$ EBV-CTLs. A, representative CFSE-based proliferation assay of control and IL-7Rα.CAR-GD2$^+$ EBV-CTLs. Control and IL-7Rα.CAR-GD2$^+$ EBV-CTLs were activated in the presence of autologous irradiated LCLs and either IL-2 or IL-7. CFSE dilution was evaluated on day 7 using FACS analysis. The graph represents mean ± SD of five independent experiments. B, representative coculture experiment in which control and IL-7Rα.CAR-GD2$^+$ EBV-CTLs were cocultured with CHLA-255 GFP-tagged tumor cells (at ratio 1:2) in the presence of IL-2 or IL-7. Residual tumor cells were enumerated by flow cytometry on day 7 of culture. The graph shows mean ± SD of five independent experiments. *, $P < 0.001$.

Finally, a CFSE-based dilution assay showed that Treg only proliferated after polyclonal activation in the presence of IL-2 and not on exposure to IL-7 (MFI 1439 ± 207 vs. 445 ± 68, respectively; $P < 0.001$; Fig. 3D).

IL-7 supports the proliferation and effector function of IL-7Rα.CAR-GD2$^+$ EBV-CTLs in the presence of Treg

Having demonstrated that IL-7 supports the proliferation and function of IL-7Rα.CAR-GD2$^+$ EBV-CTLs, we then investigated whether the beneficial effects of IL-7 were maintained in the presence of functional Treg. As illustrated in Figure 4A, when IL-7Rα.CAR-GD2$^+$ EBV-CTLs were cultured with CHLA-255 cells (effector:target ratio of 1:2) they significantly controlled the growth of these tumor cells by day 7 of culture in the presence of either IL-2 or IL-7 (residual cells were 6% ± 1% and 4% ± 1%, respectively). In contrast, when expanded Treg were added to the coculture (ratio CTLs:Treg 1:1), the antitumor activity of IL-7Rα.CAR-GD2$^+$ EBV-CTLs was significantly inhibited in the presence of IL-2 but not of IL-7 (residual cells in culture, 14% ± 3% vs. 7% ± 2%, respectively; $P < 0.05$). In addition, IL-7 also supported the proliferation of IL7Rα.CAR-GD2$^+$ EBV-CTLs in the presence of Treg upon physiologic costimulation with autologous LCLs. As the CFSE dilution assay shows in Figure 4B, the proliferation of IL-7Rα.CAR-GD2$^+$ EBV-CTLs in response to IL-2 (68% ± 4%) was significantly compromised in the presence of Treg (to 34% ± 6%; $P < 0.01$). In contrast, when IL-7 was added to the culture, IL-7Rα.CAR-GD2$^+$ EBV-CTLs divided well even in the presence of Treg (proliferation was 63% ± 3% without Treg and 56% ± 2% in the presence of Treg). The CFSE dilution of IL-7Rα.CAR-GD2$^+$ EBV-CTLs cocultured with Treg was significantly increased in the presence of IL-7 as compared with IL-2 ($P = 0.005$).

IL-7 supports the *in vivo* antitumor activity of IL-7Rα.CAR-GD2 EBV-CTLs even in the presence of Treg

To assess the *in vivo* capacity of IL-7 to support the antitumor activity of IL-7Rα.CAR-GD2$^+$ EBV-CTLs, we used NSG mice engrafted intraperitoneally with the FFLuc$^+$ cell line CHLA-255. As shown in Figure 5, control mice that received only tumor cells or control CTLs showed a rapid

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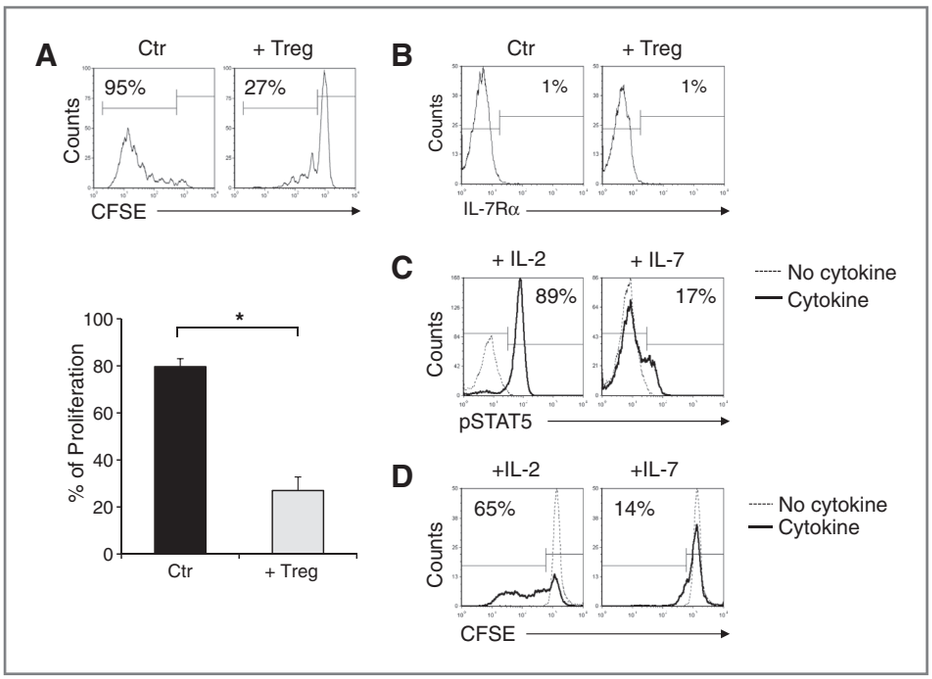

Figure 3. *Ex vivo* expanded Treg do not respond to IL-7. A, CFSE-based assay to illustrate the inhibitory activity of *ex vivo* expanded Treg. PBMCs labeled with CFSE were activated in the absence (left) or in the presence of Treg (right) at a ratio of 1:1. CFSE dilution was measured on day 7 of culture by flow cytometry. The graph represents mean ± SEM of six independent experiments. B, expression of IL-7Rα in *ex vivo* expanded Treg in a representative experiment. The plot on the left shows the isotype control, whereas the plot on the right shows the IL-7Rα profile. *, $P < 0.001$. C, phosphorylation of STAT5 in Treg not stimulated (dotted lines) or stimulated with IL-2 (left) or IL-7 (right). D, proliferative response of Treg exposed to IL-2 or IL-7. Treg were labeled with CFSE and stimulated in the presence of IL-2 (left) or IL-7 (right). CFSE dilution was evaluated on day 7 by flow cytometry. The solid and dotted lines represent the CFSE dilution of Treg stimulated with or without cytokines, respectively.

increase of the bioluminescence signal ($2.3 \times 10^8 \pm 3 \times 10^7$ photons) and were sacrificed by day 18. Mice infused with IL-7Rα.CAR-GD2$^+$ EBV-CTLs and IL-2 had superior tumor control ($1.6 \times 10^8 \pm 2 \times 10^7$ photons at day 34), but this effect was abrogated when Treg were coinfused ($2.4 \times 10^8 \pm 4 \times 10^7$ photons at day 34; $P < 0.05$). In contrast, mice infused with IL-7Rα.CAR-GD2$^+$ EBV-CTLs and IL-7 controlled tumor growth equally well in the absence ($1.2 \times 10^8 \pm 3 \times 10^7$ photons) or in presence of Treg ($1.3 \times 10^8 \pm 6 \times 10^6$ photons) at day 34.

Discussion

The adoptive transfer of CAR-redirected EBV-CTLs safely induces tumor regression in patients with neuroblastoma and the approach is potentially applicable to other human malignancies (8, 9). To further improve the clinical benefits of this approach, we developed a strategy that selectively promotes the *in vivo* expansion of CAR-redirected CTLs without favoring the proliferation and function of Treg that may limit long-term persistence and activity of the infused effector cells and thereby compromise antitumor efficacy. Here, we demonstrate that CAR-redirected EBV-CTLs engineered to regain responsiveness to IL-7 by restoring their expression of IL-7Rα proliferate in response to a combination of native T-cell receptor (TCR) and IL-7 stimulation without favoring the expansion and function of Treg. As a consequence, we observed an increase in their CAR-mediated antineuroblastoma activity, even in the presence of Treg.

Successful clinical outcome following adoptive transfer of tumor-specific T cells strongly correlates with the *in vivo* survival and proliferation of these cells (18, 32, 33). In addition to the intrinsic properties of T lymphocytes, such as central-memory versus effector-memory versus naïve phenotype that directly dictate the self-maintenance capacity of tumor-specific T cells (34), several tumor-associated mechanisms are also pivotal in determining the consequences of administering tumor-specific T cells (10, 35). Treg in particular are abundant in the tumor microenvironment and are a major factor in impairing T-cell function. Hence, strategies that selectively increase persistence and expansion of adoptively transferred T cells or that eliminate the influence of this cell subset should be as relevant for T-cell therapies as they have proved to be for cancer-vaccine trials (36).

The administration of recombinant cytokines or the use of cytokine-engineered T cells (30, 37, 38) that selectively support T-cell growth without providing functional or proliferative advantages to Treg represent appealing approaches to overcome the inhibitory function of Treg within the tumor microenvironment. However, IL-2 that is frequently used to sustain the *in vivo* proliferation and persistence of adoptively transferred CTLs is nonselective, stimulating both tumor-specific effector T cells and Treg, as both these cell

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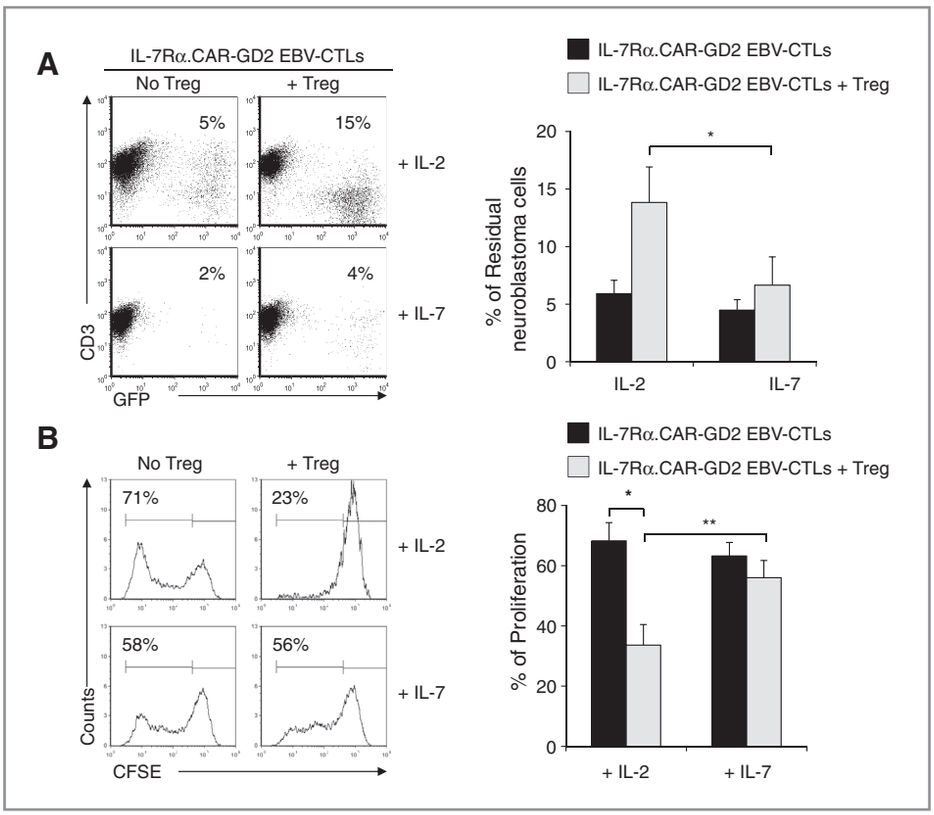

Figure 4. IL-7, unlike IL-2, supports *in vitro* the proliferation and function of IL-7Rα.CAR-GD2$^+$ EBV-CTLs in the presence of Treg. A, IL-7Rα.CAR-GD2$^+$ EBV-CTLs were cocultured with CHLA-255 GFP-tagged cells (ratio 1:2) in the presence of IL-2 or IL-7, with or without Treg. The percentage of residual tumor cells was measured by flow cytometry on day 7 of culture. The plots on the left show a representative experiment, whereas the graph on the right summarizes mean ± SD of five independent experiments. B, IL-7Rα.CAR-GD2$^+$ EBV-CTLs were labeled with CFSE and activated with autologous LCLs in the presence of IL-2 (top) or IL-7 (bottom) with or without Treg. CFSE dilution was measured at day 7 of culture by flow cytometry. The plots on the left show a representative experiment, whereas the graph represents mean ± SD of five independent experiments. *, $P < 0.01$; **, $P = 0.005$.

subsets express the IL-2 high affinity receptor (CD25; refs. 19, 39). Thus, as illustrated in the current and prior studies, the net effect of IL-2 administration is to block the proliferation and antitumor effects of CAR-redirected CTLs both *in vitro* and *in vivo*.

Although IL-7 shares several functions with IL-2, it also has effects on specific T-cell subsets that depend on their expression of the private IL-7Rα subunit (23). Our experiments demonstrate both *in vitro* and *in vivo* that IL-7 can nonetheless support the survival, expansion, and effector function of CAR-redirected EBV-CTLs if these cells are engineered to reexpress the IL-7Rα and that it can thereby overcome the inhibitory effects of Treg. Our approach has significant advantages over the use of IL-2 or cytotoxic drugs to eliminate Treg in a nonselective manner (40) as it may promote the long-term persistence of CAR-redirected EBV-CTLs both in steady-state conditions and in a lymphopenic environment (23). In addition, the administration of recombinant IL-7 unlike recombinant IL-2 seems to be well tolerated even at high doses (20, 21, 41). Finally, as

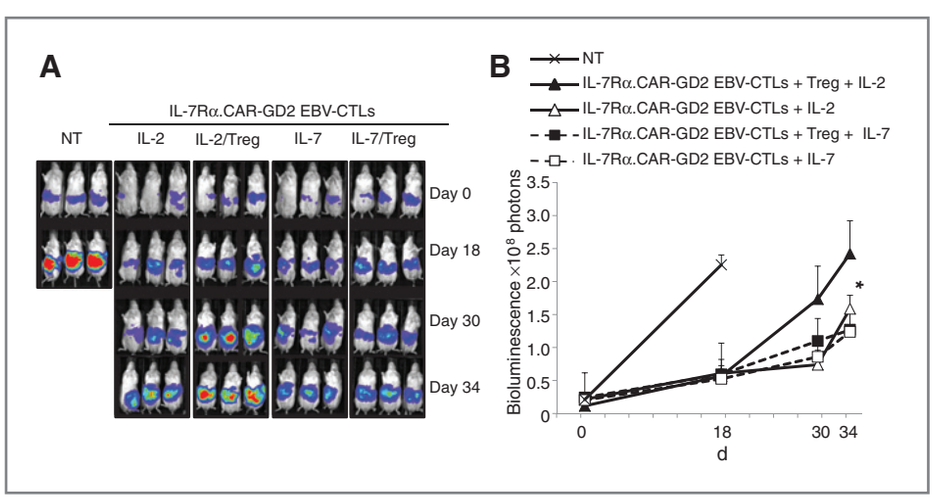

Figure 5. IL-7, but not IL-2, supports *in vivo* antitumor activity of IL-7Rα.CAR-GD2$^+$ EBV-CTLs in the presence of Treg. NSG mice engrafted intraperitoneally with CHLA-255 cells tagged with FFLuc were infused with IL-7Rα.CAR-GD2$^+$ EBV-CTLs and received IL-2 ± Treg or IL-7 ± Treg. Tumor growth was monitored using an *in vivo* imaging system (Xenogen IVIS imaging system). A group of mice received control EBV-CTLs or tumor cells only (Control). A, images of different groups of mice. B, mean ± SD of photons for 8 mice/group in two independent experiments. *. $P < 0.05$.

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the infusion of virus-specific CTLs after allogeneic stem-cell transplant does not induce the occurrence of graft versus host disease (28), our proposed approach of infusing CAR-redirected CTLs with restored responsiveness to the homeostatic cytokine IL-7 may significantly increase the application of CAR technology in the allogeneic setting (42).

One potential concern associated with any genetic manipulation of T cells is that the cells will undergo malignant transformation, or grow in an antigen independent manner. This concern is particularly prominent when the genetic manipulation modifies a growth factor receptor or other portions of a signaling pathway. However, the experience of our own and other groups has been that the genetic manipulation of differentiated T cells to express cytokines or cytokine receptors does not affect the antigen specificity of these cells and does not elicit uncontrolled proliferation (24, 37, 43). These results were confirmed in the current study even if we cannot completely exclude the possibility of secondary paracrine effects due to the ectopic expression of IL-7Rα. If such a concern remains, however, incorporation of suicide or safety switches within the cells may provide a further level of reassurance (37, 44).

Our study suggests that restoring the responsiveness to IL-7 of virus-specific CTLs redirected with a CAR is a strategy that may allow enhanced T-effector cells without concomitant inhibition by Treg and may thereby further improve the clinical outcome of a promising therapeutic approach.

Disclosure of Potential Conflicts of Interest

M.K. Brenner is a consultant/advisory board member of Bluebird Bio. The Center for Cell and Gene Therapy has a research collaboration with Celgene and Bluebird Bio. GD, BS and MKB have patent applications in the field of T cell and gene-modified T-cell therapy for cancer. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: S.K. Perna, M.K. Brenner, B. Savoldo, G. Dotti
Development of methodology: S.K. Perna, B. Savoldo, G. Dotti
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.K. Perna, D. Pagliara, B. Savoldo, G. Dotti
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.K. Perna, D. Pagliara, H. Liu, B. Savoldo, G. Dotti
Writing, review, and/or revision of the manuscript: S.K. Perna, M.K. Brenner, B. Savoldo, G. Dotti
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.K. Perna, M.K. Brenner
Study supervision: B. Savoldo, G. Dotti
Provided technical assistance for some of the *in vitro* and *in vivo* experiments: A. Mahendravada

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Review

Chimeric antigen receptors (CARs) from bench-to-bedside

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ABSTRACT

Chimeric antigen receptors (CARs) combine the antigen specificity of an antibody with the biologic properties of T lymphocytes. While the concept has been developed more than 20 years ago, only in recent years the clinical application of this approach has produced remarkable objective clinical responses. In this brief review, we outline some specific aspects that have led to antitumor responses in cancer patients.

1. Introduction

Gene transfer of αβT-cell-receptor (TCRs) and chimeric antigen receptors (CARs) can reproducibly and efficiently redirect the antigen specificity of polyclonal T lymphocytes, thus overcoming the tedious process and frequent failures of strategies based on the *ex vivo* reactivation and expansion of T-cell precursors with native antitumor activity.

CARs are composed of a specific antigen binding moiety, obtained from the variable regions of a monoclonal antibody, linked together to form a single chain antibody (scFv), and of signaling components derived from the ζ chain of the TCR/CD3 complex and from costimulatory molecules [1,2]. T lymphocytes expressing a CAR bind to the specific antigen expressed on target cells through the scFv segment and then activate their lytic and costimulatory pathways promoting cytotoxic activity and cell expansion (Fig. 1). The immediate obvious advantage of this technology is the MHC unrestriction of the cytotoxic activity mediated through the CAR component as the antigen recognition is antibody mediated [1]. This approach licenses T cells to recognize a great variety of tumor cell types as reviewed elsewhere [3,4]. Here we will briefly summarize some results obtained so far from clinical trials and indicate some future directions.

2. Methodologies to express CARs and to expand T cells *ex vivo*

Gamma retrovirus and lentiviruses are frequently used to insert CARs in T lymphocytes for clinical applications [5–10]. Such vectors have the benefit of efficiently infect T lymphocytes, integrate into the host genome and produce robust expression of the gene in human T cells and their progeny. These gene delivery systems allow rapid manufacturing (2–3 weeks) of CAR-modified T cells for clinical use [5,7–10]. Although these vectors have shown a very good safety profile when applied to T lymphocytes, concerns remains related to the potential insertional mutagenesis they have produced in hematopoietic stem cells (HSC) [11–13]. Other reservations of these vectors may reside in their limited cargo capacity and high manufacturing costs. Alternatives to viral vectors build on the delivery of plasmid DNA [14,15]. While the electroporation of expression plasmids has been essentially abandoned by the great majority of investigators due to its inefficiency, an emerging method takes advantage of combining transposone/transposes (Sleeping Beauty [14] and Piggybac [15]), with the convenience of considerably reducing the manufacturing costs and increasing the cargo capacity of the vector, favoring the inclusion of multiple genes. Still, *ex vivo* cultures required to manufacture CAR-modified T cells with this technology remain considerably long (4–5 weeks) because of the need to enrich the small percentage of CAR-modified T cells. Conversely, increasing evidences suggest that the duration of *ex vivo* cultures, required to produce sufficient number of CAR-modified cells for adoptive transfer, is particularly relevant. Preclinical models [16] and data from patients infused with *ex vivo* expanded tumor infiltrating T lymphocytes show a direct correlation between short culture conditions and increased *in vivo* proliferation/survival of these cells after adoptive transfer [17].

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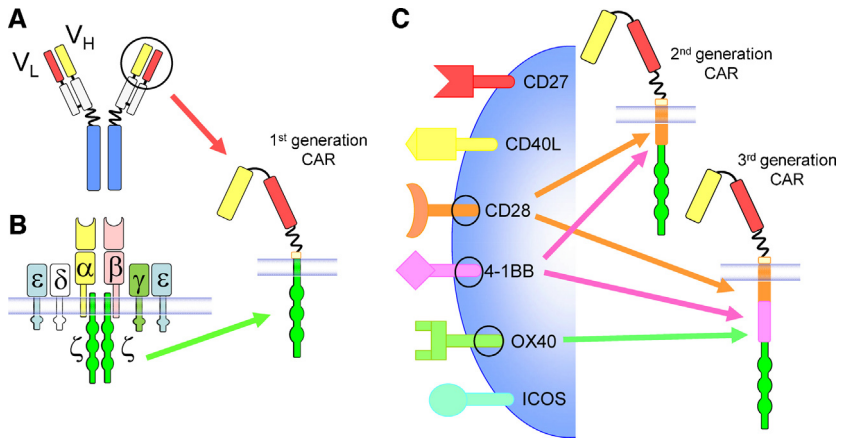

Fig. 1. Chimeric antigen receptors (CARs). CARs are most commonly created by joining the heavy- and light-chain variable regions of a monoclonal antibody (*Panel A*) that binds to a specific antigen to the intracellular portion of a T-cell signaling molecule, such as components of the TCR-associated CD3 complex (ζ-chain) (*Panel B*). Endodomains of costimulatory molecules such as CD28, 4-1BB and OX40 (*Panel C*) are then included in tandem to generate second and third generation CARs (*Panel D*).

Hence it is critical to develop methodologies that enable the generation of large numbers of CAR-modified T cells in a relatively short period of time. Finally, the cytokines employed *ex vivo* for T-cell expansion appear to affect the *in vivo* outcome of manufactured CAR-T cells. For instance, the use of gamma-chain cytokines such as IL-7 and IL-15 as opposed to the conventional IL-2 may aid in preserving subsets of T cells with central-memory characteristics, thereby favoring their long-term persistence [18].

3. CAR-modified T cells and the role of the costimulation

T-cell activation requires TCR engagement and co-stimulation provided by professional antigen presenting cells [19]. A multiplicity of sequential T-cell costimulatory receptor-ligands occurs in secondary lymphoid organs. In contrast, tumor cells and the tumor microenvironment are deficient in costimulatory signals but abundant in inhibitory factors, and ultimately induce T-cell anergy, exhaustion or death [20].

To supply costimulation within the tumor microenvironment, costimulatory signaling domains derived from molecules like CD28 [21,22], 4-1BB [23] or OX40 [24] have been incorporated in tandem into CARs (Fig. 1). This modification is undeniably a key element for the current clinical success of CAR-T-cell therapies in lymphoid malignancies. Side-by-side comparison of CARs with or lacking these endodomains clearly outlined the specific role of costimulation in promoting the persistence of CAR-T cells *in vivo* after adoptive transfer [5]. The costimulation provided by 4-1BB seems particularly effective [7,10], although additional and larger studies are needed to establish its potential superiority as compared to the CD28-mediated costimulation and its provision of robust persistence and antitumor effects also in the context of solid tumors, which are particularly abundant in inhibitory mechanisms.

4. CAR-engraftment in specific T-cell subsets

The expression of CARs in polyclonal activated T cells remains the most practical procedure used to rapidly generate large number of these antigen-specific T cells. Recently, interests have been focused on expressing CARs in specific T-cell subsets to either take advantage of the specific biologic properties or tissue tropism of each subset, or to reduce potential side effects associated with the insertion of CARs in otherwise quiescent T-cell subtypes. In this regard, CARs have been inserted in γδT lymphocytes [25], natural killer cells (NKs) [26], central-memory T cells [27,28] virus-specific cytotoxic T lymphocytes (CTLs) [6,29,30] and natural killer T cells (NKTs) [31]. γδT lymphocytes may be particularly suitable for applications in patients with epithelial tumors due to their intrinsic tropism to these tissues, while NKs and NKTs may be particularly effective in the context of the allogeneic Hematopoietic Stem Cell (HSC) transplant as they do not induce graft *versus* host disease. At our institution, we extensively investigated the use of virus-specific CTLs as a platform for CAR engraftment. In particular, we demonstrated in patients with relapsed refractory neuroblastoma that Epstein–Barr virus (EBV)-specific CTLs engrafted with a CAR can persist long term, as they receive physiologic costimulations though their native αβTCRs engaging EBV-epitopes presented by professional antigen presenting cells, while promoting objective tumor regression through the CAR component [6]. Since these cells also lack alloreactivity, we recently extended this approach in the allogeneic setting by infusing donor-derived virus-specific CTLs engrafted with a CD19-specific CAR in patients with B-cell derived malignancies relapsed after allogeneic HSC transplant [32,33].

5. Toxicities of CAR-modified T cells

Recent clinical trials have indicated that CAR-T cell-based therapies can be associated with important side effects. An obvious toxicity is related to the lack of discrimination between tumor cells and normal tissues when the selected antigen targeted by CAR-T cells is deliberately a lineage restricted antigen. An example of this type of toxicity is the depletion of the B-cell lineage by CAR-T cells redirected against the CD19 antigen. In fact, in parallel to promoting remarkable antileukemia effects, T cells directed against this antigen cause profound and durable depletion of normal B cells [8–10]. This expected side effect can be compensated by the infusion of human gamma globulins and may represent an acceptable toxicity in patients with otherwise incurable leukemia. However, now that the clinical efficacy of these therapies has been proved, the selection of more restricted antigens sounds like a realistic alternative to control the tumor growth while preserving at least part of the normal B-cell compartment [22,34,35].

Life threatening toxicities due to the concomitant cytotoxic activity of CAR-T cells on normal tissues have been reported in clinical trials targeting the carboxyl-anhydrase-IX (CAIX) [36] or HER2 [37]. CAR-T cells specific for CAIX, an antigen frequently overexpressed in clear cell renal carcinoma, have induced liver toxicity as the same antigen is also expressed by bile duct epithelial cells [36]. Similarly, CAR-T cells specific for HER2 may have target the low level of HER2 expressed in the pulmonary parenchyma or vasculature in an infused patient with lung cancer, causing fatal

pulmonary dysfunction [37]. In addition to these toxicities strictly related to the tissue distribution of the antigen, a systemic inflammatory response syndrome (SIRS) or cytokine storm has been reported in patients infused with CAR-T cells [7,10]. This effect is likely attributable to a general perturbation of the immune system and associated with the release of high levels of proinflammatory cytokines, such as TNF-α and IL-6. Although potentially reversible by the prompt administration of blocking antibodies this syndrome remains a major concern for the large scale application of these therapies to non specialized centers.

As we increase the potency and persistence of CAR-T cells it is likely that the inclusion of switch off gene systems will become desirable to rapidly eliminate CAR-T cells in case of severe or life threatening toxicity, or on demand to terminate their effects like to confine the B-cell aplasia associated with CD19-CAR-specific T cells. The inducible caspase9 suicide gene that we developed in our group is quite attractive for this function [38,39]. Indeed, this system induces rapid apoptosis of T cells, has reduced immunogenicity as the sequences are all of human origin and, is selectively activated by an otherwise bioinert small molecule known as chemical inducer of dimerization (CID) [39,40].

6. Conclusion and future perspectives

The objective clinical responses reported in small clinical studies in patients with lymphoid malignancies treated with CAR-modified T cells have been embraced by a general enthusiasm involving not only the scientific community but also biotech companies interested in help moving these treatments from the academic environment to a broader clinical application. Several steps remain to be refined especially in the simplification of the manufacturing process to extend the application of these technologies, but their effectiveness has been finally incontrovertibly demonstrated.

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